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## WORKING WITH THE MICROSCOPE





ANTONI VAN LEEUWENHOEK (1632-1723) of Delft, Holland.  
First and greatest amateur microscopist.

# WORKING WITH *the* MICROSCOPE

*by*

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WORKING WITH THE MICROSCOPE

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## *Preface*

**M**OST books written for the purpose of providing instructions in microtechnique fall into one of two classes. Either they are rankly juvenile and unscientific, invoking the sensational in an attempt to manufacture an interest in the subject, or they are highly professional and technical, designed for the upper collegiate level, and presupposing some classroom supervision.

There seems to be decided need for a manual aimed at that large body of serious workers who fall in between these two extremes. First we may list the amateur, a sadly abused designation too frequently giving an impression of some bungling tyro. Correctly used, the word amateur means one who takes up a study as a hobby or avocation, be this person a beginner or a skilled technician, often the equal and sometimes even the superior of any professional in knowledge and ability.

Such an amateur lacks one important aid. No matter how elaborate his equipment, he has no instructor at his elbow to demonstrate just how a given slide is to be prepared. The written word is never the equal of the demonstration; once we have seen someone else do an act correctly, it is easy to imitate and to understand. However, the next best thing is a book of instructions, and our aim in the present case has been to write a graded series of exercises in the mounting of materials for observation under the microscope. Beginning with the simplest and proceeding by easy stages to advanced operations, the explanations have been made as detailed and nontechnical as possible. The plan is to learn as you go, with the intent of training the reader from scratch or from any point he may previously have reached.

It must be emphasized that this volume does not pretend to include directions for the making of every known sort of slide; indeed, such an encyclopedia is neither possible nor desirable. Having once learned the construction of a given type of preparation, the reader may be expected to apply the same manipulations in the making of many others that fall in the same category. Nor does this book attempt to teach either the facts or the principles of the many sciences to which the microscope is handmaiden; that is the province of the numerous and splendid texts, widely available, and covering such fields as biology, bacteriology, criminology, and textiles.

In addition to appealing to the amateur working alone, it is hoped that this manual will serve the needs of classes and extracurricular clubs in the high school, junior college, teachers colleges, and lower levels of senior colleges and universities, as well as organizations such as microscope clubs, Boy Scouts, and similar groups unaffiliated with schools. The reading matter is designed for adults, from high school age onward, and the various chapters have been made progressively advanced, stimulating the user toward constantly higher attainments in both an understanding and an application of the materials and methods with which he is working.

The reader is advised to use the Index freely and refer to Chapter 17 for formulas. Consult the Appendixes and the List of Abbreviations. Above all, master each chapter as it is taken up and do not go on to the next until you are satisfied with your results. When in doubt as to the degree of proficiency attained, ask for a criticism of your slides by a college or high school teacher of biology, or buy a professionally made slide of the same subject from a supply house, for comparison.

Little of the material in these pages is original; what merit the volume possesses depends primarily on organization and presentation, together with illustrations that often go far toward overcoming the handicap of the written explana-

tion of some performance. Most of the methods are time-honored and to be met with in numerous manuals. In particular cases, acknowledged at the appropriate place, we have borrowed extensively from a number of standard works.

Among those to whom we feel especially indebted for cooperation and for permission to include copyright material are the University of Chicago Press, for techniques described in Guyer's *Animal Micrology* and Chamberlain's *Methods in Plant Histology*; the Collegiate Press, Ames, Iowa, for excerpts from Becker and Roudabush, *Brief Directions in Histological Technique*; Bausch & Lomb's *Educational Focus* for illustrations; the General Biological Supply House, Inc., Chicago, for various formulas; and Ward's Natural Science Establishment, Inc., Rochester, New York, for loan of the slides from which photomicrographs were made. Every volume of this nature depends a great deal also on Lee's *Microtometist's Vade-Mecum*, now edited by Gatenby and Painter and published by The Blakiston Company, Philadelphia, and on the journal, *Stain Technology*, edited by H. J. Conn. Our thanks to all of these authors and firms.

Professor Robert T. Hance of Duquesne University, Pittsburgh, gave generous permission to reproduce a number of his new procedures. The RCA Manufacturing Company, Camden, New Jersey, provided an illustration of their famous electron microscope, while leading optical companies furnished illustrations of instruments, including Bausch & Lomb, Spencer, Zeiss, Leitz and Reichert (Pfaltz & Bauer). Owing to conditions in Europe at the time of publication we were unable to extend this representation.

Most of the photomicrographs appearing in these pages are the work of John V. Butterfield, while the pen drawings were designed by Veronica E. Corrington.

JULIAN D. CORRINGTON.



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## *Abbreviations Used in This Book*

The abbreviations indicating measurements include both singular and plural, as hr. is either hour or hours, ft. is foot or feet.

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A	—alcohol, percentage of. Throughout the book such phrases as 70 per cent ethyl alcohol, or 70% alcohol, are rendered as 70A.
C.	—centigrade.
cc.	—cubic centimeter.
c.p.	—chemically pure.
E.F.	—equivalent focus.
F.	—Fahrenheit.
Fig.	—figure (illustration).
ft. or '	—foot.
g.	—gram.
hr.	—hour.
in. or "	—inch.
min.	—minute (of time).
ml.	—milliliter.
mm.	—millimeter.
mu or $\mu$	—micron.
N.A.	—numerical aperture.
oz.	—ounce.
p.	—page.
R.I.	—refractive index.
sec.	—second (of time).
U.S.P.	—United States Pharmacopeia.
wk.	—week.
X	—magnification, as 10X means an enlargement of ten times a linear dimension.



## WORKING WITH THE MICROSCOPE



## CHAPTER I

### THE MICROSCOPE

*In This Chapter:* parts of the compound microscope and their use; setting up the microscope for work, focusing, proper illumination; care of the instrument and of the eyes; definition and kinds of microscopes; the science of microscopy.

**M**ICROTECHNIQUE deals with the preparation of material of one sort or another so that it may be studied under the microscope to the best possible advantage. The operations are many and varied and will claim our attention throughout the following chapters; just at this point, however, we are mindful of certain readers for whom the microscope and its employment will constitute a new experience. These will need first of all an introduction to the microscope itself, together with some indication as to kinds of magnifying instruments and the many purposes for which they may be used.

Before going into the matter of defining a microscope, let us analyze the make-up of the variety that most people have in mind when the word is mentioned—the regulation biological compound microscope. Certain details that follow will apply only to particular models, but the general description will fit all ordinary types.

The instrument as a whole (Fig. 1) may be divided into two parts—mechanical and optical. The *mechanical parts* are secondary, but necessary for the ease of operation and the exactness of results. There must be a *stand*, the framework for all other parts, defined farther on. This rests on a *base*, the favorite design for which is a horseshoe, having three points of contact with the table top and using the familiar principle of support of the tripod. Rising from the base is the *pillar*; at the top of this, in the more expensive

types, is an *inclination joint* which permits tilting the remainder of the instrument at any desired angle, often useful as a matter of comfort but having no effect otherwise, hence not an essential feature

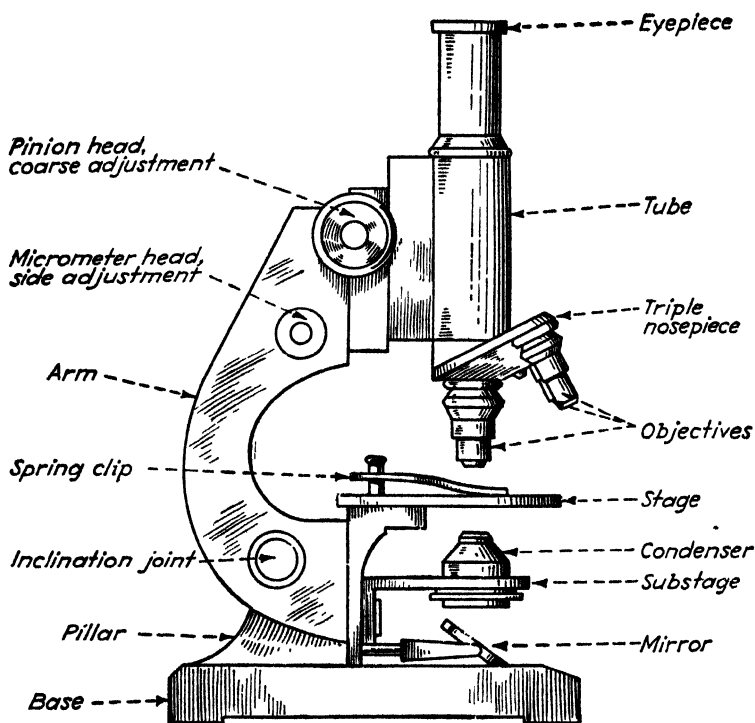


FIG. 1.—The compound microscope.

Jutting forward from the top of the pillar is the *stage*, which is truly named, since it is here that the many actors, living or inanimate, will be placed to perform for your magnified vision. A central opening or *stage aperture* allows for the transmission of light rays from below, and two *spring clips* are customarily provided in order to hold a slide securely in position. In the more costly instruments there is a *substage* beneath, carrying a *condenser*, which is actually a secondary microscope for the purpose of focusing light rays upon the specimen. In all cases there will be a *mirror*,

usually with one surface plane and the other concave. In laboratory models this reflector is supported by a *mirror arm* or *bar*, swinging like a pendulum, and having at its end a *mirror fork*, rotating vertically, while the mirror itself turns within the fork on a horizontal axis. Thus, with three different motions available, the mirror may be set quickly in any desired position or at any angle.

Immediately beneath the condenser and mounted as one of its parts is a very important accessory, the *iris diaphragm*. Like the same appliance on a camera shutter, this consists of a number of very thin pieces of curved metal, operated by a lever so as to squeeze down the opening almost to the vanishing point. In microscopes of standard size that have no condenser, either an iris or a disk diaphragm is generally mounted immediately beneath the stage aperture and serves to regulate the amount of light admitted to the specimen. The *disk diaphragm* is a revolving wheel containing a series of holes of regularly decreasing diameter, any of which may be aligned with the stage aperture. It gives the same effects as an iris but is somewhat less flexible. Miniature microscopes have no diaphragm; to make up for this omission, we can endorse the ingenious idea sent us by several correspondents, who in one way or another have contrived to fasten an old camera iris to the underside of the stage.

Rising above the inclination joint is the *arm*, which is that part of the stand carrying the *tube*, also known as the *body* or *body tube*, the carrier of the optical parts. In order to focus the lenses, this tube must be capable of vertical motion, for which purpose there are generally two wheels. The *coarse adjustment*, sometimes the only one present on cheap models, operates by a rack-and-pinion gear and changes the elevation of the tube rapidly; the knurled wheel grasped by the operator for this action, generally one on each side, is the *pinion head*.

If a *fine adjustment* is also present, it is actuated by a smaller knob, the *micrometer head*. There may be only one



of these heads, located in older instruments at the top of the arm and revolving horizontally, when the arrangement is termed a "top fine adjustment." If situated at the side of the arm, below the pinion head, and revolving vertically, the style is a "side fine adjustment," with either single or double micrometer heads. In many of the better models the right-hand one of these heads bears a micrometer scale so that the exact amount of vertical motion may be determined. Further, the fine adjustment may have definite upper and lower limits, reaching either of which it refuses to turn farther, or it may operate on an endless, reversing gear. In the high-priced instruments, movement of this adjustment ceases as soon as the objective touches the cover glass over the specimen, thus preventing damage to either object or lens. The fine adjustment moves the tube up and down very gradually and so permits accurate focusing, but it is necessarily one of the most expensive parts of the stand and is dispensed with in miniature or other of the less expensive forms of microscope. In some amateur instruments, the same service is performed by a button that moves the stage instead of the tube, effecting a marked saving in cost of construction.

The tube is a hollow cylinder bearing the objective at the lower end and the eyepiece at the upper. In some models, one objective must be unscrewed and removed before another can be put in position; in others higher power is gained by adding one objective lens directly upon the first one; in still others, there is a *revolving nosepiece* carrying two or more objectives so that lenses of different powers may be swung into line as required, and all of them protected from dust whether in use or not.

In some instruments there is a *drawtube* at the top of the body tube, pulling out like the sections of a small telescope. This act increases the magnification.

So much for the mechanical parts, all of which except the tube and its accessories are collectively referred to as the *stand*, previously mentioned. The *optical parts* include the

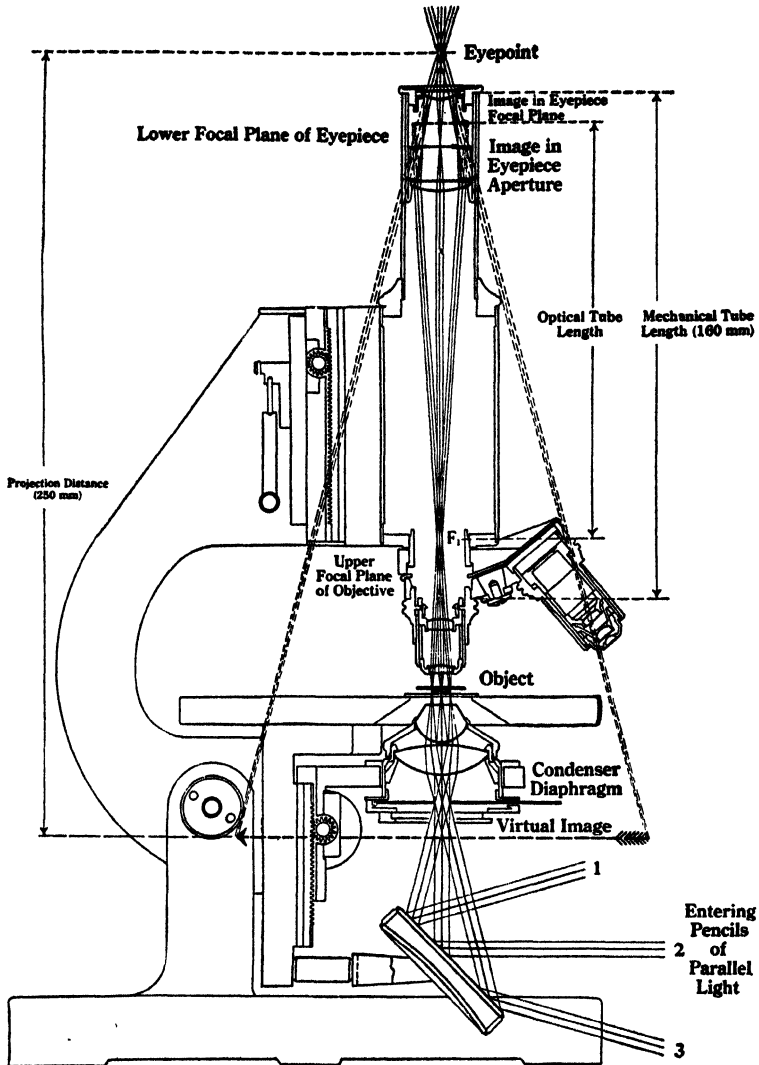


FIG. 2.—Path of light rays through a compound microscope. (Courtesy of Bausch & Lomb.)

mirror, condenser (when present), objective, and ocular, all of which must be in perfect alignment or, in other words, lie along the *optical axis* of the microscope. The aperture and specimen must also be placed on this axis, which is that of a beam of light in passing from the mirror surface through the aperture, specimen, and lenses to the eye (Fig. 2). The eye itself is an optical instrument, with its own focusing lens and sensitive recording plate or retina, and forms an integral part of the whole system whereby magnified images of objects are recorded in the brain.

The *objective* is so called because it is situated next to the *object*, the specimen being examined. The *eyepiece* or *ocular* is that part nearest the eye of the *observer*. Object, microscope, and observer are hence the triple alliance for all studies of the very little. In this book we shall devote the lion's share of our time to the most variable as well as the most easily altered of these three elements—the object.

The commonest type of laboratory microscope is equipped with two oculars and two objectives. The shortest objective in any given series is always the lowest in magnifying power; the reverse is true of oculars, the longest ones being the lowest in power. Since any eyepiece can be used in conjunction with any objective, if two of each are present, four combinations are possible. The more elaborate stands are equipped with a triple nosepiece carrying three objectives, the additional one being an *oil immersion* lens, designed for use only when connected to the cover glass over the specimen by a drop of especially purified cedar oil.

Most users of microscopes are interested in questions of magnification and will wish to know how the various powers are designated. For many years there was no universal standard for indicating the performance of an eyepiece or an objective, and even today not all manufacturers use the same schemes. The earliest markings were arbitrary symbols, as 1, 2, 3; I, II, III; or A, B, C, the first in each such series denoting the lowest power. When optical instruments came into general use, however, there

arose a demand for some form of designation that would give information as to relative merits, and one of the first standards adopted was the *equivalent focus* (abbreviated E. F.), engraved on the side of the brass shell housing the objective lenses. This convenient invention treats the lens combinations of an objective as if they were a single, simple lens, and means that at some selected distance the several lenses within an objective will work together to give the same magnification as would a simple lens of a certain designated focus.

Inasmuch as the component lenses of an objective must be shaped and spaced with particular distances in mind at which they are to be used, an *image distance* of 250 mm. and a *tube length* of 160 mm. were agreed upon by the majority of manufacturers (Fig. 2). At this image distance, a simple lens of 25-mm. focal length yields a magnification of 10  $\times$ ; consequently any compound objective, which at the same distance gives the same enlargement, 10  $\times$ , is said to have an equivalent focus of 25 mm. Thus a designation for the objective, E. F. 25, which would really tell something about its performance, became available.

Older American optical products were so marked in inches, and British makers still adhere to this system. More recently American factories have adopted the Continental method, using the metric system. For example, the standard low power objective found on most microscopes was formerly marked  $\frac{2}{3}$ —the E. F. in inches; now it would bear the legend 16 or 16 mm., the metric equivalent. Still more recently, it has become customary to include the actual magnification as well, labeling the part "10  $\times$ ," a procedure that should have been done in the first place. The high power objective is similarly indicated as  $\frac{1}{6}$  (in.) or 4 (mm.), 43  $\times$ . This magnification is that of the objective alone and is termed the *initial magnification*.

Eyepieces, after the early A, B, C stage, were generally engraved 1 and 2, the E. F. in inches. Today the 1 is marked 10 and the 2 is 5, the magnifications. The *final*

*magnification* of any combination being used is determined by multiplying that of the objective by that of the ocular; thus, the 10 eyepiece with the 43 objective gives an enlargement of 10 by 43 or 430.

The following table shows these features in the most frequently used achromatic combinations, based on an image distance of 250 mm. and a tube length of 160 mm. If your microscope has no drawtube, the tube length is built in and unchangeable, and will be designated by the maker in his catalog or booklet of instructions; if a drawtube is present, you will find a scale upon it by means of which the correct tube length may be obtained.

Equivalent focus		N. A.	Initial magnifying power	Final magnification with Huygenian eyepieces				
In.	Mm.			5 ×	7.5 ×	10 ×	12.5 ×	15 ×
2	48	.08	2	10	15	20	25	30
1½	32	.10	4	20	30	40	50	60
¾	16	.25	10	50	75	100	125	150
⅓	8	.50	21	105	157.5	210	262.5	315
⅙	4	.65	43	215	322.5	430	537.5	645
⅛	1.9	1.25	97	485	727.5	970	1,212.5	1,455
⅜	1.8	1.30	100	500	750	1,000	1,250	1,500

Another point concerning magnification: Some of the less scrupulous advertisements are misleading in their representations of "power," and such statements put the knowing buyer at once on guard. Magnifying power should be designated in terms of diameters only and not in areas. Thus a 10 × lens is one that makes a line appear to be ten times longer than without magnification. Since a square would be enlarged ten times along one side and ten along the other, the included area would be one hundred times as great; it would give an entirely false idea to state that, for this reason, such a lens had a power of 100 ×. Any one dimension is magnified only ten times.

## USING THE MICROSCOPE

Having digested the foregoing anatomy and physiology of the microscope, the beginner is now in a position to proceed intelligently. For the first studies he will need a few blank microscope slides and at least one prepared slide or permanent mount of some object, such as a fly's leg, wing, or proboscis, butterfly wing scales, a flea, a section of plant stem, fine print, textile fabric, or any of dozens of similar subjects. Various items of glassware, dissecting instruments, chemicals, and pieces of apparatus are mentioned throughout this volume, their nature and use being explained when first introduced. Wherever feasible, pointers are given that will assist in the home construction of many of these; in Appendix A will be found a list of supply houses from whom these requisites may be purchased. Very often it is possible to substitute something already at hand or in the home; in any case it is expected that the average reader will make or acquire these materials a few at a time so that no considerable expense at any one period is involved.

For pointers on the operation of your particular microscope, be sure to study the booklet of instructions provided by the manufacturer.

**To Focus the Microscope.**—Remove the microscope from its case, grasping it by the arm. Carry it to the work table and place it down gently, remembering that it is a precision instrument that will not be improved by banging or careless handling. Never carry it upside down as then the eyepiece is apt to fall out and smash on the floor. Place it on the table squarely in front of you, mirror facing away from you, and sit at a proper height so that you can look down the tube without craning upward or hunching downward; the body erect, the neck bent so as to lower the head over the eyepiece.

Raise the tube by turning the coarse adjustment toward you so that there is plenty of clearance between the stage

and the objective; then mount a prepared slide with the cover glass up. This is the small circle or square of very thin glass under which the specimen is mounted. Center the object directly over the stage aperture, the slide held in place by the spring clips, which should not come in contact with the delicate cover glass. The microscope body may be inclined or vertical, as desired, using the inclination joint if one is present. Looking to one side and not down the tube, adjust the mirror, plane surface up, so that a bright light shows on the object; then, with the low power objective swung into the optical axis, lower the tube until within about  $\frac{1}{4}$  in. of the slide.

Now for the first time look down the tube. Probably the first thing to do will be to make further adjustments of the mirror in order to secure proper and uniform illumination. To do so, grasp the edges of the mirror with the thumb and forefinger of both hands and turn and twist so that the mirror surface is aimed directly at the light source and reflects a bright circle of light upward to the stage aperture, where it should be centered and without dark areas, high lights, bars, or other hindrances—evenly illuminated throughout. Begin by having this circle of light, termed the *field* of the microscope, as bright as possible; then operate any diaphragm present, or insert the frosted or blue glass disk with which some instruments are equipped, so as to cut down the brightness to a moderate amount. Tissue paper may be used for this purpose, or the microscope may be withdrawn farther from the light source. More about illumination later on.

Grasp the pinion head with the right hand and very slowly *raise* the tube until the object comes into view. Sharpen the image by using the fine adjustment, if one is present. This method is termed *focusing up* and, if always followed, will never result in broken slides or lenses. Never run the tube downward while looking through it, as it is easy to flash past the focal point and continue downward until an accident happens. Focusing upward is one

of the signs by which to judge the properly trained microscopist.

Operate the fine adjustment so as to determine that the structure being examined has a definite thickness—that you can look at the bottom surface, the top surface, and the levels in between. As a test object to demonstrate this matter of depth of focus in a very convincing fashion, take an inch each of three differently colored threads, as red, yellow, and blue. Cross these over one another on a blank microscope slide, the threads radiating outward like the spokes of a wheel from the common center where they cross. Add a drop of water and a cover glass; then examine them under the low power objective, then the high power, focusing on the place where the three threads cross. A permanent balsam mount may be made if desired by putting on a drop of balsam instead of water, as explained more fully in Chapter 3.

Next choose some object that has recognizably oriented parts, such as a flea, with its head and tail ends, back and belly surfaces. Observe that the object is seen upside down (inverted image); therefore, if it has such parts, it should be mounted upside down when the slide is placed on the stage so that it will appear right side up through the microscope. Move the slide and note that the speed of motion as well as the size of the object is magnified, and that all motions are reversed. You have to move the slide upward (away from observer) if you want to see more of the upper parts (apparent lower parts), or toward the left to see parts off the left edge of the field (apparent right edge). This would seem to cause endless confusion, but after a bit of experience these reversed motions become automatic and natural.

If your microscope does not have a nosepiece, it will be necessary, in order to change objectives, to raise the tube upward quite a distance to get clearance, make the necessary change, then refocus, using the same procedure of focusing up. If a nosepiece is present, you merely swing the high



power objective into the optical axis, but it is wise at first to do so very slowly, with the eye down at the level of the stage, looking through between stage and objective, to make sure that the high power lens is not going to strike the slide. It should come very close indeed, but not quite hit. Thickness of the mount on the prepared slide will make a difference; sometimes the object is too thick to be focused under high power. One has to learn his own microscope just as he would his rifle or automobile, and make allowance for individual differences.

If the high power lens does not strike the slide, this performance need not be repeated for all future mounts of ordinary thickness. Look down the tube. If the object is already in approximate focus without further adjustment, the microscope is said to be *parfocal*, and one can swing from high to low power and back again at will without doing more than possibly making slight movements of the fine adjustment to sharpen the focus. All microscopes when sent from the factory are supposed to be parfocal but, in the course of time and with the wear of mechanical parts, they may cease to be so. Often this condition may be restored by putting a collar of one to several thicknesses of paper around the base of the threads of that one of the two objectives whose focal point is too high. Manufacturers supply shims for this use, on request.

Not infrequently it may happen that the fine adjustment movement comes to a stop and no longer propels the tube. This means that it has reached the end of either its upward or downward course in those instruments not equipped with an endless reversing gear. When this occurs, reverse the movement of the fine adjustment, moving it in the direction in which it is free to travel, until somewhere near the middle of its run, then refocus with the coarse adjustment. You may now turn the fine adjustment either way to sharpen the focus.

It will be noted that the field is smaller and the light dimmer when high power is substituted for low. The

diaphragm should be opened or the amount of light otherwise increased. The rule in using the mirror with a condenser is to employ the plane surface at all times; without a condenser, use the plane surface for low powers, the concave for high powers.

### Microscope Lamps.—

Next to the microscope itself, nothing is of greater importance than proper lighting, for light of some sort is the medium by which we observe the object. Getting the right kind and amount of light will have much to do with what we see and how well we see it.

Daylight is frequently employed; for such use the work table should be set across some window and both microscope and observer face the light. Adjust the mirror, plane surface in use, and direct it upon fleecy white clouds or, if none are present, upon clear blue sky. Never focus on or near the sun, which is far too bright.

There are times and places where daylight cannot be employed, as in dark rooms, during cloudy or stormy days, or at night. For this reason and to secure an ever uniform and dependable light, most workers prefer an artificial source in the form of a microscope lamp. Generally this light is directed up through the object from below, when it is termed *transmitted light*. However, one may wish to study opaque objects, as coins, bullets, or pieces of rock, or observe the surface features of animals, plants, or textiles; in such a case the light is directed down upon the subject from above and is called *incident* or *reflected light*.

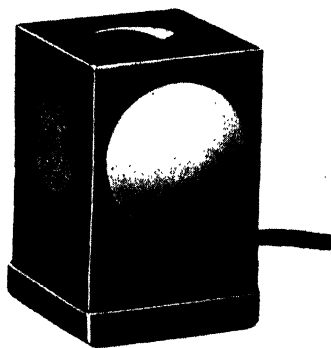


FIG. 3.—Substage lamp. (Courtesy of Bausch & Lomb.)

The *substage lamp* (Fig. 3) is a very popular box-like type which can be stood in front of the mirror or, with mirror removed, laid flat between the feet of the microscope base, if the latter is of the horseshoe design. Intensity of the

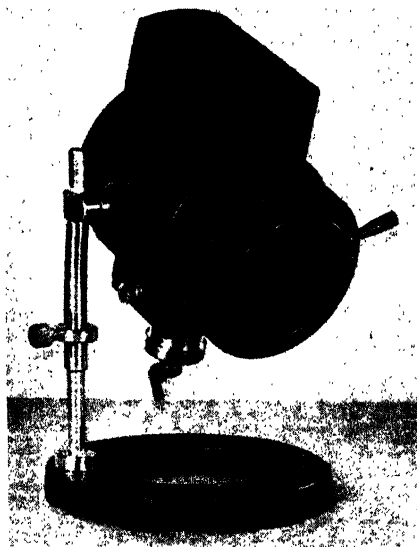


FIG. 4.—Adjustable microscope lamp equipped with strong illuminant, condenser, diaphragm, and filters. (Courtesy of Spencer Lens Company.)

light is easily controlled by varying the distance between the lamp and the mirror. Other models available commercially are more complex, some including a bull's-eye lens to focus light rays on the substage mirror, and even a rack-and-pinion focusing gear (Fig. 4). Still other lamps utilize an electric arc, especially necessary for high power photomicrography.

Many amateurs prefer to make as much of their own equipment as possible and a lamp is one of the more easily constructed appliances. A coffee can makes a good one (Fig. 5). Discard the lid and in the center of the bottom cut out a circular piece of such a diameter that the hole will fit a lamp socket snugly. Punch half a dozen small holes through the sides, near the bottom, equally spaced, for ventilation, then cut

out a semicircle in the side and at the top, providing a window through which the light is to come. Paint the exterior if desired, but leave the interior bright. Wire and insert the socket, add a 25- to 40-watt opal or frosted bulb, and turn the can bottom end up for use.

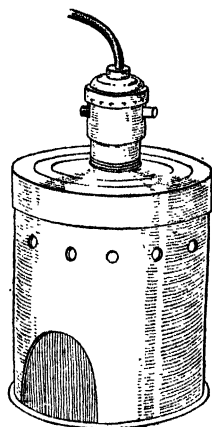


FIG. 5.—Coffee-can microscope lamp.



FIG. 6.—Baking-powder-can lamp.

A somewhat different model may be built from a baking-powder can (Fig. 6). Attachment of the socket follows the foregoing plan, but support is furnished by a short length of brass curtain rod, driven into a wooden base. The latter may be round or square, and cut from the end piece of an orange crate, painted or stained if desired. Hammer and file the upper end of the brass rod flat and drill it to receive a small bolt. Cut a strip of tin from a second can and wrap this around the waist of the electric socket, above the threads, leaving two tab ends of  $\frac{1}{2}$  in. each, which are then drilled to take the bolt. This sleeve now permits the attachment of the lamp to the stand, as well as adjustment at any desired angle. Paint the exterior of the can any preferred color.

The lid may be used merely to keep out dust when the lamp is idle, but some owners of this style of lamp have rigged up the lid to hold either or both condensing lens and color filters. To do this, cut out a central opening about one-half the diameter of the lid, but leave several projecting tab ends which may be bent inward to hold a lens or filter in place, across the opening and against the inside of the lid. Filters may be made of colored cellophane, blue glass, or ground glass, and either secured to the can lid or mounted in a small rack to be stood between the lamp and the microscope. By saving a number of lids from the

same variety of can, one may fasten a different material to each and then secure different effects by changing lids. Old flashlight lenses make fairly good bull's-eye condensing lenses, though better ones may be bought from an optician for a small sum. Many microscopists use a flask of water colored a fairly deep blue by the addition of copper sulphate crystals and stand this between the lamp and the microscope, where it serves as both condenser and filter.

For studies by reflected light, some miniature microscopes are arranged so that the operator removes the mirror from below and plugs it into the arm, above the stage. For other models, use a student gooseneck reading lamp, brought over the specimen as far as possible; rig a small flashlight, fountain-pen style, alongside the microscope tube; or wire a cheap mirror to the microscope body or to a separate stand placed alongside. There are professional vertical illuminators, some of them housed inside the microscope tube, but they are all rather elaborate and expensive and would be indicated only if one were making many and detailed researches needing this sort of lighting.

**To Clean the Microscope.**—For keeping lenses and slides clean there is a choice of several materials, every microscopist having his own preferences. Some use prepared lens paper, obtainable in loose sheets or in book form; others like an old linen handkerchief, made soft through repeated laundering. Still others think that nothing is better than a small piece of chamois skin. Personally, we belong to the old-handkerchief school and save our worn-out ones for this purpose.

Before starting any particular observation, clean both surfaces of prepared slides by breathing on them and polishing quickly. If this does not suffice, wash the slides in water, taking care not to wet the labels. Next clean the mirror surfaces and top lens of the eyepiece, and also wipe gently the lower surface of the objective. If a condenser is present, this part will need more thorough and frequent cleaning than any other, as it is a great dust catcher.

From time to time, a more extensive cleaning is in order. Take the eyepiece apart and clean both surfaces of each of the two lenses. Never take an objective apart, however. Dust and grease cannot get into the interior of an objective; should there ever be any evidence that something is amiss, send the part back to the factory, since only experienced technicians with proper equipment can clean or adjust the delicate setup of small lenses in an objective.

Do not handle glass surfaces with the fingers as this always results in oily prints. If it is necessary to remove grease, oil, glycerin, balsam, or other such substances which have accidentally got upon the lens surfaces, use a soft rag moistened with benzene, then wipe this solvent off promptly so that it will not damage the setting of objective lenses. Never use anything harsh or any of the strong cleaning mixtures recommended for other types of glassware.

In the case of oil immersion objectives, wipe the oil from both lens and slide as soon as you are through with a given study. After the oil has caked or hardened from failure to clean promptly, use a rag moistened with xylene.

If the rear surface of an objective needs cleaning, as it may occasionally, use a camel's-hair brush, which is also excellent to remove lint and dust from other glass parts. Always keep an eyepiece in place in the body tube, since this prevents dust from falling on the rear of the objective.

Looking down the tube, with the microscope focused on a slide, move the slide back and forth. Everything that moves is on the slide, and one can thus readily tell whether or not the slide is clean and free from dust, grease, or other foreign substances. Next twirl the eyepiece while it is resting in place; everything that rotates with this action is on one or more surfaces of the eyepiece lenses and will indicate whether further cleaning is necessary.

Keep the microscope covered when not in use. For this purpose there is the case in which you received it—supplied with most instruments—as well as cloth hoods, made or

purchased, and bell jars of cellulose acetate, a new product far cheaper than glass. Dust is the greatest enemy of the microscope; use a camel's-hair brush and a soft cloth or chamois and keep the stand and all mechanical parts free from dust. Reserve a discarded toothbrush for use on the teeth of the rack-and-pinion gear. Once in a while, remove the body from the arm and thoroughly clean all exposed parts of this gear, with brush and cloth; then with a very small quantity of pure paraffin oil on a bit of cloth, wipe the slides on which the tube operates so as to oil completely, then go over them again and wipe away all excess oil. The rule is to oil as sparingly as possible, since oiled surfaces catch dust and gum up readily. Do not put any oil on the teeth of either the rack or the pinion.

In similar fashion, using a toothpick, occasionally place a very small drop of this oil on the shank of the pinion head on each side, to lubricate the coarse adjustment. Do not, however, attempt to care for, take apart, adjust, or otherwise experiment with the fine adjustment of an expensive microscope; should it require attention—which is rare—return it to the factory. It is too complex and delicate for the attention of anyone save an expert.

Do not use alcohol or any strong chemicals on the stand as they will probably damage the lacquered finish. Above all, keep the microscope clean and free from dust and avoid banging or any sudden jars. With reasonable care a good instrument should last a lifetime.

**Rules for Care of the Eyes.**—1. Use the correct amount of light. This means a moderately bright light, regulated (*a*) by having a proper source of light to begin with, (*b*) by the setting of the mirror, (*c*) by varying the distance between light and mirror, (*d*) by using glass or paper filters to dim the light, and (*e*) by operating the diaphragm. The light should be strong enough so that one does not strain the eyes in attempting to see, but not so bright as to produce any glare. Strong light is not only bad for the eyes—it also

drowns out all detail of the object under examination. Vary the amount of light and notice the corresponding variation in the amount of detail that can be seen. One quickly learns the right light to use under different conditions.

2. Keep *both eyes open*! This is absolutely necessary, and is a bit of a trick to learn at first. What happens in the beginning is that you will see the field of the microscope for a moment, then it will blur or vanish and you will see the table top with the other eye, only to see the field again a bit later. Practice at this a little at a time—not over 2 or 3 min. without a rest—and not over 10 or 15 min. at one sitting, two or three times a day. Within a short time you will train the eyes so that only the one looking down the tube will record an image, the other will see nothing. Patience and practice; keep at it; it will come.

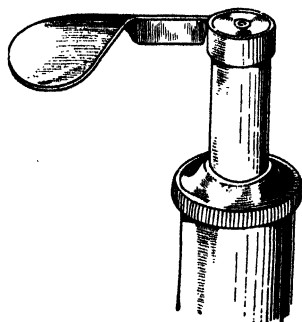


FIG. 7.—Light shield for beginners while learning to keep both eyes open.

Keeping both eyes open is one mark of the trained microscopist. Only the beginner or the poorly advised student shuts one eye and squints down the tube, just as one sees the beginner at target shooting with a rifle do the same thing. Not only is this method less effective, but the squinting tires and harms the eyelid muscles and induces eyestrain. Sometimes beginners employ a shield (Fig. 7) to shut off one eye. This may be purchased or is easily made from black photomount cardboard and is useful during the learning stage; but it should soon be discarded.

3. Alternate the use of your eyes. Train both of them, one after the other. Look down the tube with the right eye for a while—15 min. of a working period—then shift to the left eye for an equal period. This prevents unequal strain and the development of a “microscope eye.”



4. Alternate microscope study with some other occupation, as reading a reference or manual, attending to staining or other technique, or drawing. It is neither advisable nor necessary in most cases to look steadily into the microscope for more than a few minutes at a time. One naturally turns from scrutiny of, say, a new protozoan, to looking him up in a book, making a sketch drawing of what is seen, or writing notes, then looks back into the microscope again to check the first observations, and so on indefinitely. This procedure is not only excellent to prevent eyestrain, it is the natural and efficient method of working. Once in a while, it may be necessary to make prolonged observations, as when one catches a cell in division or watches fertilization or some other significant happening; but such occasional sessions do no harm.

5. Relax. Let the microscope do the work. This is a most important rule to follow and is possible only if all parts of the microscope are clean and the instrument in sharp focus. When so adjusted, there is nothing for the eye to do but look—no necessity to squint or strain—nothing to be gained by striving to see something that is not there. If one can get himself into the frame of mind that he is looking down into a deep well and that the object is far away, which is actually the case if you consider relative sizes, the eye will accommodate itself to focus at a distance in a naturally relaxed position, and the lens muscles will not be strained in a futile attempt to accommodate to a close-up view. Again, let the microscope do all the work; that's what it's for. Don't squint; don't strain.

This is quite an array of rules and you can hardly be expected to master them all the first day. But keep them in mind and, before you know it, these acts will become second nature and require no further conscious efforts. If these rules are followed, you can study with the microscope all through a long life without any impairment of vision or harm to the eyes; if disobeyed or ignored, you may have difficulties.

## KINDS OF MICROSCOPES

The variety we have been discussing is a member of a large family, with brother and sister microscopes of many different designs, as well as cousins that employ similar principles but go under other names. The most generally used of basic optical appliances are the mirror to reflect light rays, the prism to bend them, and the lens to bring them to a focus and thus form an image. The more elaborate instruments, such as our compound microscope, utilize various combinations of these fundamental units.

A *microscope* is an optical instrument that furnishes an enlarged image of some object at close range. The object itself need not be small; one may study a skull, a tree stem, or a machine with a microscope; yet the immediate part scanned is always a very small area of the whole and the image will present minute details invisible to the eye alone. Commonly, too, the microscope is directed upon entire objects, such as protozoa, bacteria, spores, and crystals, that cannot be observed at all without magnification.

Microscopes are divided first of all into two major classes: simple and compound. The distinction is not on a basis of number of lenses, as is popularly supposed, but on their design and arrangement in connection with the kind of image produced.

**Simple Microscopes.**—These may consist of one or several lenses that require the cooperation of the eye in order to form an image on the retina, the sensitive recording plate of the human optical instrument. The picture thus produced is erect and exactly like the original object save that it is larger. It is termed a *virtual image* because it has no real existence apart from the eye.

The oldest of all magnifying devices is a single lens, one of a pair of spectacles intended to aid faulty or failing vision. Those of us who wear glasses carry a pair of simple microscopes astride our noses without thinking of them as

such. Other familiar examples of single-lens simple microscopes are the reading glass and various hand magnifiers, such as those used by the stamp collector or for the examination of fingerprints.

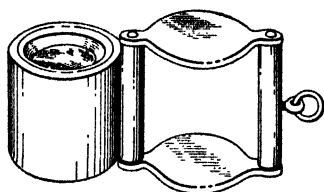


FIG. 8.—Hand lens.

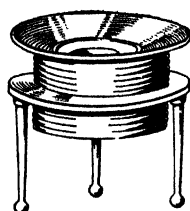


FIG. 9.—Tripod magnifier.

Images formed by single lenses are, however, subject to several kinds of distortion; in order to correct at least the most serious of these, two or more separate lenses may be combined. Sometimes they are ground so as to fit tightly together, cemented with balsam, with an action equivalent to that of a single lens, as we have seen in discussing equivalent focus. Each part is then termed a *component*. Or the lenses may be separated by more or less space; in such a case two lenses make up a *doublet*, three a *triplet*. Without exception, however, all such instruments produce only a single virtual image.

The pocket lens or hand lens (Fig. 8) is a doublet or triplet with a wide variety of uses, both in the field and in the laboratory. The tripod magnifier (Fig. 9) is a large-sized doublet housed in a three-legged frame, the glass screwing in or out for focusing when standing over a specimen on a table, or picked up either with or without the frame and employed as a hand lens. There are adjustable lens holders (Fig. 10) of many styles, the chief advantages being exactness of focus and freedom of both hands for manipulation of instruments; a box type of school dissecting microscope (Fig. 11) with fixed mirror, glass stage, tool compartment, and a tripod magnifier held in a sliding, swinging arm; and lastly the finest development of this

sort of optical aid, the *dissecting microscope* (Fig. 12), with a heavy metal stand, substage mirror, glass stage, and spring clips, rack-and-pinion coarse adjustment, removable hand rests, and a swinging, hinged arm carrying the

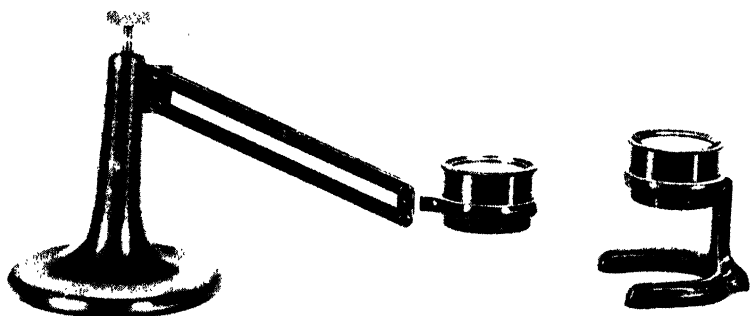


FIG. 10.—Wide-field magnifier (left) mounted in parallel arm, the lens remaining horizontal at all elevations. The same lens (right) in horseshoe base, focusing by screwing in and out. (Courtesy of Bausch & Lomb.)

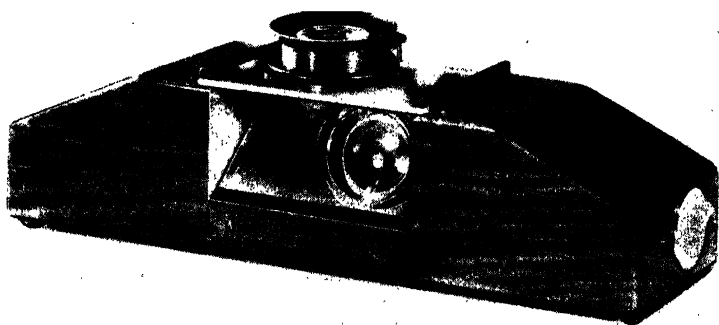


FIG. 11.—Bausch & Lomb box-type dissecting microscope, a simple and inexpensive accessory.

magnifier. The best for general purposes is the  $10\times$  aplanat. A metal plate, black on one side and white on the other, may be slipped under the glass stage to provide suitable backgrounds for variously colored objects. This form of microscope combines many of the advantages of the compound instrument with very low power and is extremely

useful in the study of many classes of subjects, as embryos, parasites, small invertebrates (notably insects), fruits, flowers, seeds, textiles, metal objects, and so on through a long list of materials for which low magnification is often

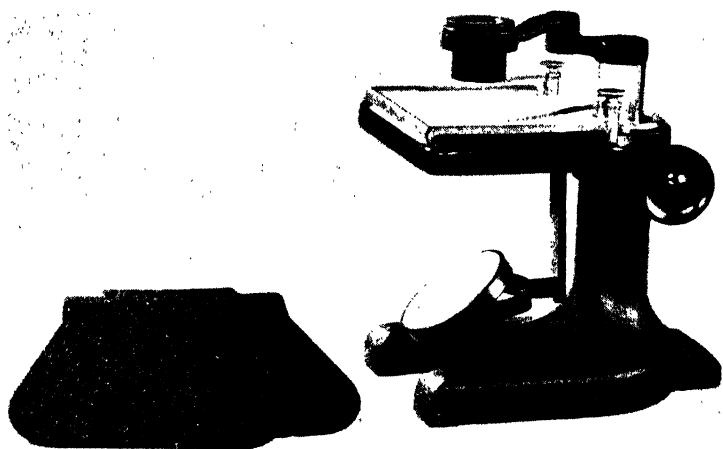


FIG. 12.—Spencer dissecting microscope. Objects at left, on table, are detachable hand rests.

very desirable. As the name indicates, this instrument is especially serviceable for fine dissections of all sorts.

Certain other types have been devised for special uses. The watchmaker's loupe fits into and is held by the socket of one eye, or is equipped with a headband, in either case leaving both hands free for work. Binocular loupes are spectacles that provide both eyes with a low power lens. The linen tester folds to fit the vest pocket or opens to provide the correct focus in counting the number of threads per inch in textiles. Engraver's glasses and fingerprint readers are further variations of the tripod magnifier theme.

**Compound Microscopes.**—These are also made in many different forms for special purposes. They all differ in principle, however, from the simple microscope in that two

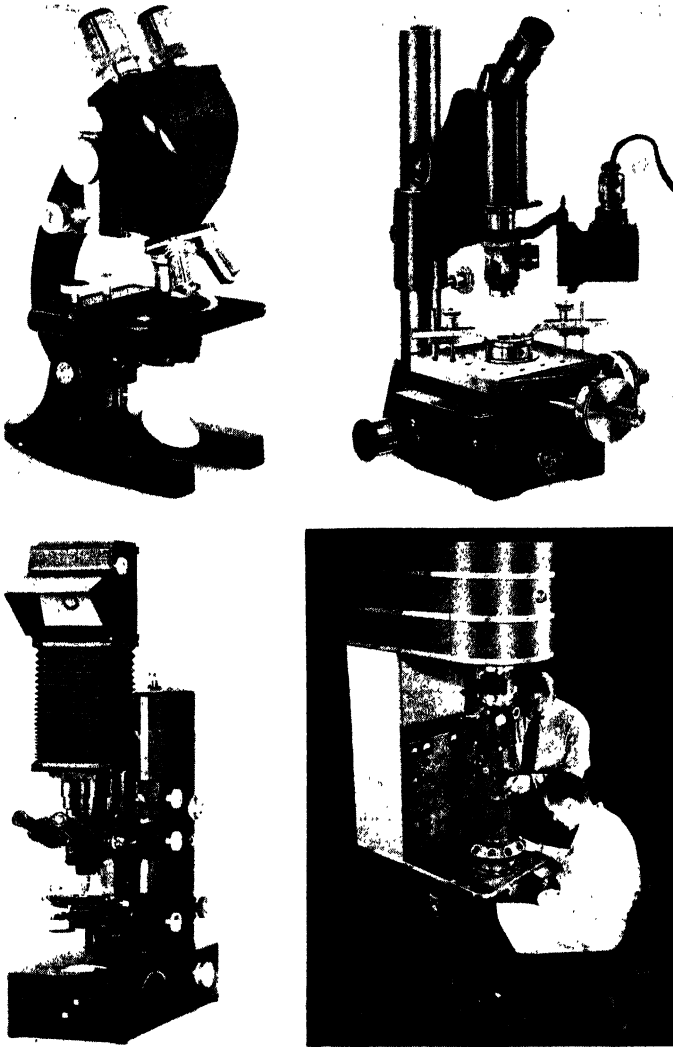


FIG. 13.—Some modern, specialized types of microscopes. Upper left, Bausch & Lomb research instrument with interchangeable inclined binocular body, triple nosepiece, mechanical stage, and substage condenser; upper right, Bausch & Lomb toolmaker's microscope, including inclined erecting monocular eyepiece, vertical illumination, and positioning chucks on mechanical stage; lower left, Zeiss Ultraphot universal microscope with camera, binocular eyepieces, and built-in light sources for every form of illumination; lower right, RCA electron microscope.

images are produced. The objective forms an inverted *real image* of the object; this in turn is scanned through the ocular which, like a simple microscope, gives an erect virtual image and further enlargement. The arrangement is that of one microscope on top of another; hence the term "compound." A real image has an independent existence, regardless of whether or not there is a human eye present to see it, and makes possible the arts of photography and projection. A real image may be thrown upon a sensitized plate and there captured as a photograph, or it may be projected upon a screen from a lantern slide or motion-picture film.

With many of the variations in the general plan of a compound microscope the differences are found in the stand rather than in the optics. The medical microscope includes appointments specified by the majority of American medical colleges and represents a better grade of instrument than the ordinary laboratory model. The nosepiece is triple, mounting a 16-mm. low power dry, a 4-mm. high power dry, and a 1.9-mm. oil immersion objective. Two eyepieces, 5 $\times$  and 10 $\times$ , are supplied, and there are an Abbe substage condenser and a built-in mechanical stage, the movements of which are controlled by milled heads. Research microscopes go still further in the incorporation of refinements. They have a heavier and sturdier stand, better fine adjustment and condenser, and the most perfect optics. A recent idea is an interchangeable body whereby the usual monocular may be replaced by a binocular, either erect or inclined (Fig. 13).

The wide-field binocular microscope (Fig. 14) is actually a double instrument, with paired eyepieces and objectives that give a stereoscopic effect, bringing the third dimension of depth into strong relief. Necessarily expensive because of the use of paired and matched optics, this is by far the finest of all low power instruments for use with relatively large objects such as those listed in describing the dissecting microscope.

Petrographic microscopes (Fig. 15), also termed "chemical microscopes," are designed for the study of rocks, minerals, and crystals in geology, mineralogy, and chemistry and incorporate a number of parts that bring about a

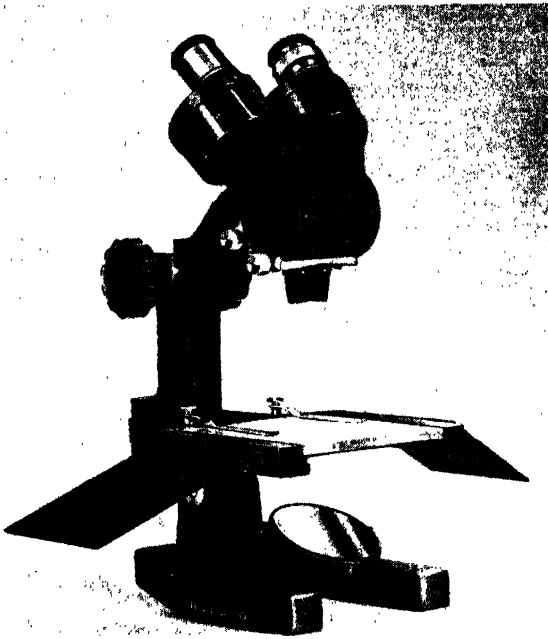


FIG. 14.—Wide-field binocular microscope with drum-type nosepiece housing three sets of paired objectives. (*Courtesy of Bausch & Lomb.*)

quite different appearance. The stage is circular and revolving, extra parts may be inserted or withdrawn from the tube, and elements permitting the use of polarized light are built in.

The comparison microscope (Fig. 16) has two tubes and objectives, but the two images are brought side by side into the field of a single eyepiece for direct comparison. Designed primarily for investigations in scientific crime detection, this instrument has found many laboratory and industrial applications. There is also available a compari-



son eyepiece so that any two similar monocular stands may be converted into a comparison microscope.

Amateur microscopes (Fig. 17) are like ordinary models but are miniature in size. Those made by recognized optical

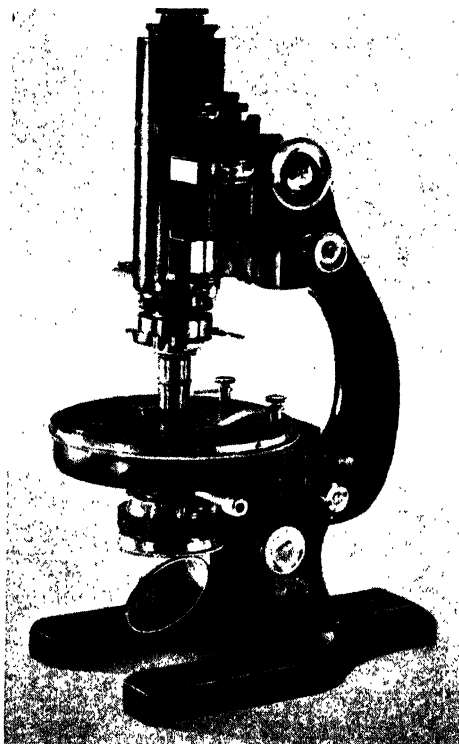


FIG. 15.—Spencer petrographic microscope.

manufacturers are sturdily built and include first-class, corrected optics, with powers up to 300 or 450  $\times$ . They are well suited to all classes of general work save bacteriology and will permit the making of good photomicrographs. Unfortunately their popularity has resulted in the flooding of the market by unscrupulous jobbers with an avalanche of worthless, junk "microscopes," sold in department stores, toy shops, and even the ten-cent store. Failure to

achieve the promised results has discouraged many a young beginner, who has turned away from microscopy as a hobby with the notion that there was nothing to it. He was not experienced enough to realize that you cannot get some-

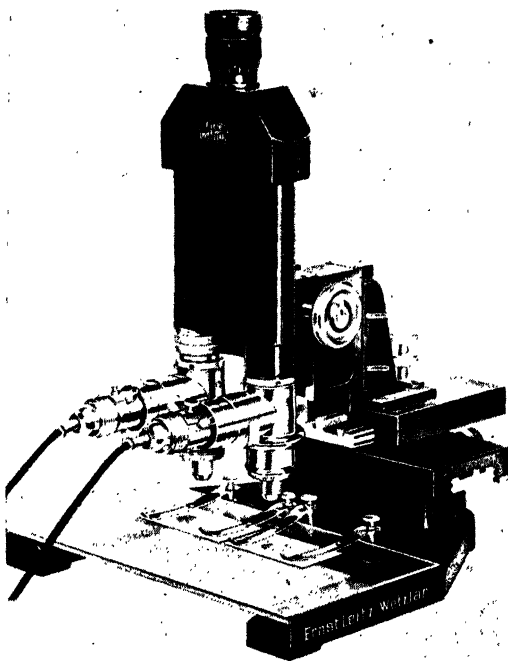


FIG. 16.—Comparison microscope equipped with vertical illuminators. (Courtesy of E. Leitz, Inc.)

thing for nothing, and that it is not possible to manufacture so complex an instrument as a microscope for a few dollars.

There are portable microscopes that can be knocked down to take afield in a small flat case; toolmaker's microscopes (Fig. 13) for measuring depressions and screw threads and for the inspection of dies, castings, and forgings; metallurgical microscopes for work with metal specimens; and projection microscopes especially designed to throw magnified images on a screen, or downward upon drawing paper so that accurate tracings are rendered easy. Still

other types go by special names. There are the colorimeter, like a comparison microscope, enabling the matching of a solution of unknown concentration with a known standard; the refractometer which measures angles of refraction of

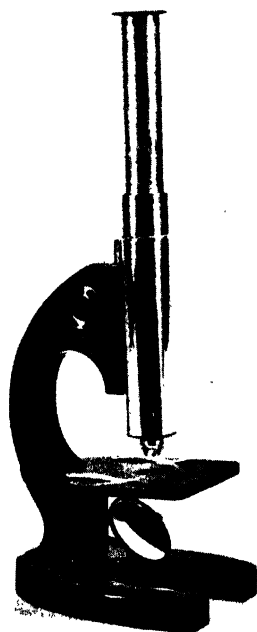


FIG. 17.--Bausch & Lomb Model R, a miniature microscope with bakelite stand.

light and thus aids in the analysis of unknown substances; dust counters, opacimeters, and glossmeters of the paper technologist; ophthalmoscopes, retinoscopes, and otoscopes of the eye and ear medical specialist; centrifuge microscopes that keep an object under observation while subjected to the tremendous forces of thousands of revolutions per minute; microdissectors so precise that a single bacterium may be removed from a mass of its fellows, a chromosome isolated from a cell, or an amoeba cut in two and its nucleus extracted; and electron microscopes (Fig. 13) that employ electron beams instead of light and so achieve, with the photographic enlargement of micrograph prints, the stupendous magnification of 150,000  $\times$ !

Akin to microscopes in general principles but differing as to construction of lenses and their spacing, are those members of the telescope family, whose function is to bring distant objects nearer through an apparent enlargement. The refracting telescope, spyglass, opera glass, binoculars, and the spotting scope of rifle matches are familiar examples, and the spectroscope is one of the most important scientific aids in analyzing the composition of all sorts of materials, terrestrial or astronomical.

Microscope objectives are made for use in a medium of air, water, or oil. The first is termed a "dry objective," the other two "immersion lenses." Water immersion objectives were formerly much more popular than at present, the perfection of oil immersion optics having largely supplanted the earlier invention.

Eyepieces are of many types and designs. That supplied with all regulation laboratory microscopes today is an adaptation of one worked out for the telescope by the Dutch astronomer, Huygens (hī'-gēnz), and hence known as the Huygenian (hī-gē'-nī-ăn) ocular. Examination of the one on your own instrument will reveal that it consists of a barrel with two lenses, one at each end, the upper being the *eye lens* and the lower the *field lens*. Take the ocular apart and observe that both lenses are plano-convex—one surface flat, the other convex—with both convex surfaces directed downward, and that the eye lens is the stronger (more convex) of the two. Situated near the middle of the metal barrel is a platform with a central opening which serves to decrease the diameter of the tube at this point. It is the *eyepiece diaphragm* and it is at this level that the real image formed by the objective is brought to a focus. The field lens works with the objective in producing this image. It slightly decreases the magnification and in so doing increases the size of the field being examined and brightens the image. An ocular in which the real image is formed between the eye lens and the field lens, as in this case, is designated a *negative ocular*.

An example of a *positive eyepiece* is the Ramsden, formerly more in use than at present. Both lenses are plano-convex but the field lens is closer to the eye lens and has its convex surface directed upward. The two function together as a simple microscope to scan the real image at the level of the diaphragm, which is below the field lens.

Compensating eyepieces may follow either the Huygenian or Ramsden design and are purposely overcorrected to compensate for undercorrection of apochromatic objectives,

the most expensive and specialized form of objective. Flat field oculars are corrected and of superior quality, to yield a flatter field than usual. Manufacturers have their own trade names for these: Hyperplane (B & L), Planoscopic (Spencer), Orthoscopic (Zeiss), and Periplanatic (Leitz). Micrometer eyepieces are equipped with a finely ruled scale for measuring objects; projection eyepieces have a long focus and low magnification, especially made for microprojection and photomicrography.

The demonstration eyepiece is one housing a prism so that emergent light rays are split into two paths, one to each of two viewing lenses. This permits two people, such as instructor and student, to observe the same object simultaneously, and a movable pointer can be operated to indicate parts of the object under discussion. Of recent introduction is the inclined eyepiece holder, inserted in the tube of any regulation microscope in order to achieve the greater comfort of inclined position yet with the tube erect, so necessary in studying fluid cultures.

Very often one of the problems that arise in using a compound microscope is that of getting a sufficiently low power for the work in hand. Higher power is easy, up to the limit of the optics available, but contrary to popular belief, more work is done with the lower than with the higher magnifications. Can one go below the lowest stated enlargement of the eyepiece and objective furnished with the instrument?

Although not recommended by manufacturers, one can unscrew and lay aside the front combination of lenses of the low power objective (16-mm. achromat), whereupon the remaining component will serve excellently as a very low power lens, focusing at a considerably higher level. Certain modern instruments are equipped with a 16-mm. objective marked as "divisible" and intended for just this use; or one may purchase 32- or 48-mm. objectives if much of this work is to be done.

Another trick is to have a pointer in the eyepiece. There may be many occasions on which you will wish to point

out some object to another person. This can be done verbally by referring to the field as if it were marked off like the face of a clock and directing attention to "that round black object near five o'clock," or "it's about halfway from the center toward half past ten." But many cases arise in which this system is inadequate; a pointer in the eyepiece is much better. Such a pointer may be purchased, or you can easily make your own.

To add this accessory to your outfit, unscrew the eye lens of the ocular and set it aside. With scissors cut a single hair, preferably from someone with fine, straight, black hair. With two forceps stretch a short piece of this hair across the center of the top surface of the eyepiece diaphragm, affixing each end to the diaphragm at opposite sides with a very tiny spot of balsam. Allow it to dry for a day; then snip the hair in two at the middle and remove one half completely, balsam and all, leaving the other end to project halfway across the diaphragm. Replace the eye lens and insert the ocular in the tube, focusing on an object. Since the hair lies in the plane of the real image formed by the objective plus the field lens, it appears to be superimposed on the object and in focus. Twirling the eyepiece will rotate the hair; this, plus moving the slide, will enable you to place the tip of the hair exactly upon any desired spot.

## THE SCIENCE OF MICROSCOPY

We shall have to contradict ourselves at the start and state, strange as it may seem, that there is no such thing as a science of microscopy! The microscope is a tool and, as such, is a means to an end, not an end in itself. Wherever an enlarged image will contribute information—and where will it not?—there the microscope can be applied. We shall have to search a long time indeed to find any field of study or research in our present generation that has not been invaded by the microscope or one of its family of instruments. Let us survey some of these fields briefly in order to become acquainted with the possibilities that lie locked up within your microscope.

Although the microscope is a tool, it is so ingenious and intricate that the machine itself provides a fascinating

subject for research; in fact, microscope design is the only division of science to which the term "microscopy" in a limited sense might be applied. More correctly, however, microscope design and use fall within the province of *optics*, which is in turn a division of *physics*; here the microscope is only one of many instruments connected with the study and utilization of light.

Before one can observe most objects under the microscope, they must be prepared in special ways, such as by cutting very thin slices of opaque organs like a human kidney or a tree root, by grinding down a rock or mineral until it is thin enough to transmit light, or by processing a film of blood. All of the many steps performed in preparing materials for observation or photographing under the microscope fall within the realm of *microtechnique*, sometimes termed *micrology*. Cutting thin sections is *microtomy*; taking photographs through the microscope is *photomicrography*.

These words come from Greek roots. *Micro* means small; *ology* is a study or science; *scope* is from *skopos* (a watcher) and denotes a watcher or viewer; *otomy* means to cut; *photo* is light; and *graph* is to write. Examples of combining forms are: *micron*, the unit of measurement under the microscope, 0.001 mm., abbreviated by the Greek letter  $\mu$ , pronounced "mu," and equivalent to the English *m*; *biology*, the science of life (*bios*); *telescope*, an instrument to see things that are far off (*tele*); *anatomy*, a science employing surgical methods of cutting. It is excellent practice in any science hobby or study to use a good dictionary freely and to learn the etymology and meaning of all technical names as they first occur. If you know their derivation, they will be remembered more easily.

The beginner should quickly learn to pronounce these words properly too, particularly the basic terms "microscopy" and "microscopist" (one who practices microscopy). In microscopy, the *i* is long, first *o* short, and second *o* long, the *y* short and clipped; accent on the second syllable, thus,

my-krahs'-kō-pī. The other form of this word is similar, last *i* short: my-krahs'-kō-pīst. These are tongue twisters at first; practice them until you can reel them off glibly.

*Astronomy* relies chiefly on the telescope and spectro-scope, modifications of the principle of the microscope. About the only use here for the microscope proper is in studying thin sections of meteorites to determine their components. *Geology* requires the microscope in order to investigate the minute structure of rocks and minerals composing the crust of the earth on which we live. *Mineralogy* is a division of this earth science, in which the chief instrument is the petrographic (*petro*, rock) microscope, arranged mainly to use polarized light in analyzing the structure of mineral crystals. This leads to *crystallography* and takes us over into *chemistry*, where crystal structure is also important, and hence into many industrial fields. Chemistry uses the microscope for analysis of minute quantities of unknown substances, by observing crystals, ash, and chemical reactions, having the advantage of needing only very small amounts, a factor that may be highly important at times when only small amounts are available, as in criminal investigation or the study of rare materials.

*Biology*, the study of life, with its twin daughters, *botany* (plants) and *zoology* (animals), comprises an enormous group of sciences, some of the chief of which are anatomy (gross structure), histology (microscopic anatomy—organs and tissues of the animal body), cytology (cells), embryology (development), genetics (heredity), evolution (group origins), physiology (function), psychology (mental functions), anthropology (study of man and his relatives), eugenics (human improvement through heredity), euthenics (human improvement through environment), ecology (relation to environment), taxonomy (classification), chorology (geographic distribution), and paleontology (time distribution by study of fossils). Each of these, save the ones applying solely to man, can be used with either plants or animals or



both. Thus there are plant ecology, animal histology, plant genetics, human physiology, and so on. Medicine, sociology, economics, and in fact all studies dealing with man are but subdivisions of biology after all, since man is himself an animal and subject to the laws regulating animal life. A broad biological background is highly important for anyone specializing in any of these multiple fields.

In addition to all of these words, the use of which you will master in time through mere repetition, biological terms are further complicated by the specialties based on subdivisions of the animal and plant kingdoms. Thus bacteriology, an immensely important science where the microscope is supreme, is a subdivision of botany in which the structure, development, behavior, heredity, and other phases of just one plant group—the bacteria—are considered. Thus we have protozoology, the science of those microscopic, one-celled animals that are of great importance in geology, ecology, and medicine, many of them causing serious infectious diseases; ornithology, the study of birds; helminthology, the parasitic worms; entomology, the insects; and so on.

In every one of these many biological sciences, the unit is the cell and, since most cells are microscopic, our instrument is of paramount value; in fact no great progress in these fields was made until after the microscope had been invented and came into more or less modern form.

However, these so-called pure sciences are by no means the only ones in the domain of microscopy. In recent years, especially, the applied sciences, embracing engineering and all of the leading industrial fields, have learned that progress must be directed by this king of all instruments, the microscope. The manufacturer of steel, leather, paper, textiles, paint, rubber, and all the other basic industrial products has come to rely on the microscope for details of structure, to improve quality and detect impurities. Drinking water, foods, and drugs must be protected, the

air we breathe analyzed, the cosmetics we use inspected. It can truthfully be said of our modern and complex civilization that everything we eat, drink, wear, or use has passed at some stage of its manufacture or marketing under the all-seeing scrutiny of the microscope. Our world could not have advanced to anything like its present stage of development without this instrument. No wonder microscopy makes the very finest of hobbies!

Latest of sciences to rely heavily upon our instrument is *criminology*, more properly, scientific crime detection. Bullet scratches, fingerprints, specimens of blood, hair, handwriting, typewriting, inks, paper, textiles, dirt, wooden splinters—these are but a few of the materials which the new scientific investigator in this province must master and which, properly analyzed, will yield absolutely incontrovertible evidence—the kind that gets convictions from judges and juries.

These, then, are some of the studies you can pursue after you learn to use the microscope and to prepare slides for observation beneath its lenses. The best approach is to sample all of these lines of work before settling down to one or a few; you never can tell at first which ones you are going to like the best. What may seem unattractive now may capture your chief interest later. So our advice for the first year of microscopy is to browse around, follow out all, or at least a majority, of the studies we shall outline for you, and make many different kinds of mounts and sections.

There is nothing to be gained, however, by continuing this desultory sort of work. After it has served its purpose of introduction and orientation, one should avoid keeping on in aimless fashion. The second year, or whenever introductory study has been completed, should find the microscopist choosing a specialty, or at least a very few allied specialties, and then settling down to intensive work. Whether it be bacteriology, criminology, or crystals, human histology, genetics, or cytology, he will get vastly more out of his

hobby—be it profession or avocation—by concentrating and going ahead with advanced studies. Into these higher fields we cannot go in this book, but our purpose will be served if we can stimulate the reader with the desire to go further, and if we have shown him how to work and where to turn for additional and more advanced information.

## CHAPTER 2

### TEMPORARY MOUNTS FOR IMMEDIATE STUDY

*In This Chapter:* types of microscope slides and cover glasses; cleaning glassware; collecting and examining microorganisms from pond water; making up solutions; alcohols; studying blood, circulation, hair, convection currents, crystallization, Brownian movement; miscellaneous objects.

**B**EFORE we can examine most objects, after having our microscope all set up and focused and with some knowledge of how to operate it and with the correct light from an adequate source, we still need one more item—a blank slide on which the object is to rest.

**Slides and Covers.**—When the word “slide” is used hereafter in this book, unless qualified in some way, it will mean the regulation 3- by 1-in. blank slide—thoroughly cleaned. It should be handled by the edges only, since fingers have a habit of leaving oily imprints on polished glass surfaces.

There are other kinds of slides too. Geologists prefer a smaller size; for some embryos and sections of large organs, such as the brain, a 3- by 2-in. size is used. The regulation type comes in several thicknesses and qualities. Some are made of white glass, others appear greenish when viewed on edge and are designated as noncorrosive. Slides are packed in boxes containing half a gross and catalog prices are quoted per gross. It is unwise to buy a smaller quantity than one box at a time, as you will need many slides in this work. So, at the outset, we suggest that you secure one ½-gross box of 3- by 1-in. noncorrosive blank microscope slides. In England slides are commonly termed “slips.”

Certain observations require a *microculture slide*, which

has one or sometimes two shallow depressions ground into the top surface, or a *deep-well slide* (Fig. 18), which is a thick slab of glass with a straight-sided central depression of considerable depth. These are purchased singly, are a bit expensive, but are used over and over.



FIG. 18.—Deep-well slide.

*Cover glasses* constitute a vexing problem for the amateur who does not care to

stock them in quantity, because there are so many different kinds. They are commonly furnished in two thicknesses, Nos. 1 and 2, though two other grades may be procured from some sources. These, with their average thicknesses, are No. 0—0.10 mm.; No. 1—0.15 mm.; No. 2—0.20 mm.; No. 3—0.25 mm. For beginners and general elementary work, we recommend the No. 2 since these may be cleaned with little or no loss from breakage, whereas the cleaning of thinner grades requires some practice to eliminate this wastage. For advanced work and with all slides intended for use with the higher powers, oil immersion, or special lighting, use the No. 1 grade. As examples, whole insects or their parts may be mounted beneath a No. 2 cover; sections of organs under a No. 1.

As to shape, there are circles, squares, and rectangles. Sizes are quoted in either inches or millimeters. Those standard for squares and circles are  $\frac{1}{2}$ ,  $\frac{5}{8}$ ,  $\frac{3}{4}$ ,  $\frac{7}{8}$ , and 1 in., corresponding to 12, 15, 18, 22, and 25 mm. Rectangles are procurable in a wide variety of dimensions and are generally reserved for such special purposes as blood smears and serial sections of embryos. When all of these factors—thickness, shape, and size—are combined, it may be seen that a very large assortment of covers is involved, and the worker must choose, from this number, the ones that will best meet his own requirements and specialties.

Covers are sold by the ounce, the unit package being the half-ounce. Circles are more expensive than squares. Also, the smaller in size and the thinner the grade, the more

covers there will be to the package. Some dealers break packages of both slides and covers and sell these by the dozen for the benefit of the beginner who wishes to conserve his funds; but one naturally pays a much higher rate for this service and in the long run it is quite uneconomical. If you are a beginner who intends to make a considerable number of the slide mounts proposed in this book, we recommend that you secure at the outset one box ( $\frac{1}{2}$  oz.) each of No. 2, 22-mm. squares; No. 1, 18-mm. squares; and No. 1, 15-mm. circles. From time to time, as stocks of these diminish, make replacements by purchasing slightly different sizes so that you will gradually build up a considerable variety and thus have just the sort of cover you need for each kind of mount.

Circles always present a neater appearance in the finished slide and should be used with all mounts that are to be sealed or placed in cells. Squares may be used, as desired, with all other types of mounts, and at a considerable saving over circles. Always choose a cover that will fully enclose the material, with some room to spare all around.

**Cleaning Glassware.**—As received from the makers, slides and covers are never absolutely clean; the best routine is to clean a whole boxful of each when first obtained, and then store them in a dust-tight receptacle. This makes a somewhat tedious session but, once attended to, the job is done for some time to come and subsequent work proceeds more smoothly, without the constant interruption of having to clean up slides and covers as needed.

A hot suds of soap flakes followed by a thorough rinsing is usually sufficient, though many microscopists prefer alcohol or ether. (*Caution:* Ether is explosive; keep it away from flame.) Some technicians store slides and covers in separate jars of alcohol, wiping them dry as needed; we prefer to dry and polish the whole lot at one sitting and store them dry in covered jars.

Old and soft linen cloths that are free from lint should be

used. Rub the slides until they are dry and polished, hold them to the light for inspection, then breathe on each side in rapid succession, and give them a final polishing. Cover glasses are easily broken because they are so thin; one needs a bit of practice in handling them and must expect to break a few at first. Drape the thumb and forefinger of the right hand with a cloth, then with the left hand place a cover glass into this fold of cloth and rub it with the right

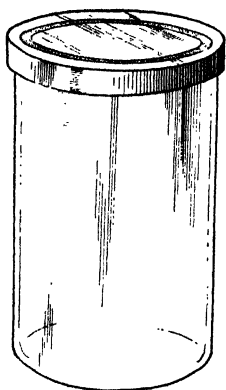


FIG. 19.—Tall stender.

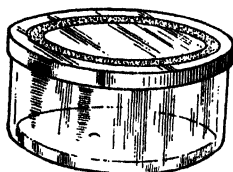


FIG. 20.—Flat stender.

thumb and forefinger in rotary fashion. This ensures an equal pressure on both sides of the glass. Another way is to hold the glass by its edges with the left thumb and forefinger while polishing with the cloth held between right thumb and forefinger, rubbing gently back and forth. Finish by holding the glass to the light for inspection, and by breathing and more polishing. Large rectangles are best polished between two cloth-covered blocks of wood.

A type of laboratory glassware known as *stender dishes*<sup>1</sup> makes excellent storage containers. There are a tall stender (Fig. 19) for storing slides, cotton, wooden splints, etc., also useful as a staining jar, and a low flat stender (Fig. 20), excellent for storing cover glasses as well as for fixing tissues and imbedding in paraffin. The lids fit within a groove that will exclude moisture and dust; always keep these jars covered except during the actual removal of contents.

<sup>1</sup> Several types of laboratory glassware have been named after the original designer or the place where first used. Thus there are Stender and Petri dishes, Coplin staining jars, and Syracuse watch glasses. In conformity with what appears to be the most recent usage, these names are not capitalized in this text.

Handle cover glasses with forceps and avoid touching clean ones with the fingers save to hold them by their edges.

Unruly glassware that refuses to polish properly and slides and covers that have once been made into balsam mounts may be cleaned up by use of a drastic formula, the bichromate-sulphuric glass-cleaning mixture, that will dissolve off almost anything. See Chapter 17, where all formulas recommended in this manual appear together for convenience of reference. The sodium metasilicate solution is another good and somewhat more general cleaner, being useful for glass, metal, porcelain, china, and agate vessels of all sorts.

#### EXAMPLES OF TEMPORARY MOUNTS FOR IMMEDIATE STUDY

With the microscope, light, and glassware in readiness, let us now make a *temporary mount*, a preparation not processed in any way to preserve it nor with the cover glass sealed on permanently. The purpose of such mounts is to study fresh material, which is then discarded; after that the slides and covers are cleaned and returned to their containers. No attempt at any sort of complete selection is possible, there being many thousands of subjects; rather what we have in mind here is to exemplify several different kinds of temporary mounts, with the idea that the reader will then be able to adapt the methods to a wide variety of additional materials.

**To Mount a Drop of Pond Water.**—Of all introductions to the fascinations of microscopic studies, none is better than the observation of some of the minute plants and animals that abound in a drop of pond water. They are utterly different, so tiny and so weird, and yet perform all the acts and functions with which we are familiar in the higher organisms. They breathe, feed or make their own food, move about, and reproduce; they are sensitive to stimuli and react to such physical forces as light and heat. Here one sees life at its very beginning. It is not surprising that college courses



in biology lay great stress on studies in microbiology, since these forms provide the starting point, if one is to understand the higher types—the old rule of proceeding from the simple to the complex.

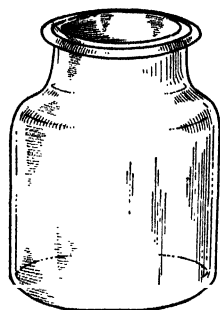


FIG. 21.—Wide-mouthed collecting bottle.

But let us first collect our specimens. One of the best sources of these is a roadside ditch containing greenish water, or a small pool, pond, or quiet eddy in a stream. Tidal pools with vegetation in them are excellent if one is collecting at the shore. City parks often provide suitable places, and the water that accumulates in ornamental urns on gateposts, in cemeteries, or in tree hollows, will harbor an interesting assemblage.

Use a squat, wide-mouthed form of collecting bottle (Fig. 21) and pass this beneath the surface of the water with the cork still in place. When on the bottom or among a growth of water plants, release the cork and allow the water to flow in, at the same time poking bits of plant material, dead leaves, soil, and other solid debris into the bottle with the finger. Replace the cork, bring the bottle to the surface, wipe it off with a cloth, and immediately attach a label giving the location and the type of water. Keep each specimen separate; do not mix collections from different places in the same bottle. Have at least ten times the volume of water to solid content in each case.

Collecting nets are described later in connection with insects. Those used in making hauls of microorganisms—protozoa, hydra, worms, rotifers, and microcrustacea among animals, and diatoms and other algae from the plant kingdom—are called *plankton nets* (Fig. 22) and are small affairs made of fine bolting silk. A net ring or wire is fastened to three or four tow lines which shortly converge into a single lead line. This net may be trolled from a boat or fastened to the end of a long stick or fishing pole so as to make casts or

sweeps from the shore or across small pools. Still better is the form of plankton net in which the conical bag of silk ends in a metal ring to which is attached a cylindrical container of fine-meshed wire screening. Organisms are con-

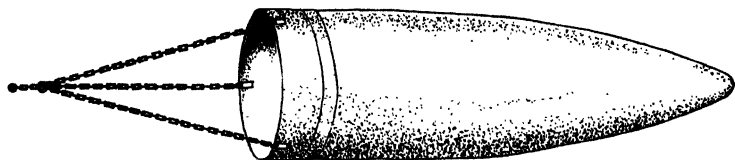


FIG. 22.—Plankton towing net.

centrated in this vessel, which is unscrewed for the removal of its contents. The plankton includes all minute plants and animals living on or near the surface of the water.

The British have long made great use of a *collecting stick*, an ordinary looking cane of bamboo that turns out to be a veritable Pandora's box of gadgets when prepared for action. The handle unscrews, a telescoped extension rod comes forth, to which can then be attached a cutting knife for underwater plants, a scraping or dipping spoon, a drag hook, a collecting bottle, or a plankton net. For some reason, this excellent and inexpensive outfit has never been taken up by American microscopists.

At the end of a collecting trip, the bottles are assembled on the work table and each cork replaced by a small square of cheesecloth, secured around the neck with a rubber band, so as to admit oxygen to the livestock within. Each bottle now becomes a *culture* of organisms, since they are to be maintained under artificial conditions.

Another method of securing material for this work is to make your own cultures by means of *infusions*, which are simply the soaking of materials in water so as to prepare living quarters in which the countless spores and cysts of bacteria and protozoa floating in the atmosphere or present in the water or solid material may develop and flourish. Among the most widely used of these preparations is the hay infusion, prepared as follows: draw off a 1-gal. *battery*

jar (Fig. 23) or a 2-qt. fruit jar of tap water and allow it to stand uncovered for 24 hr. Pond or rain water is even better if available. Then gather a small handful of dry hay or grass from any field or meadow, immerse it in the vessel of water, put a lid on the jar, and stand it in a window where it will receive light, but not direct sunlight. Another infusion is made in the same fashion by soaking lettuce leaves; still other plant materials may be

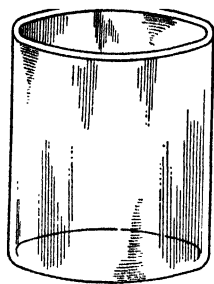


FIG. 23.—Battery jar.

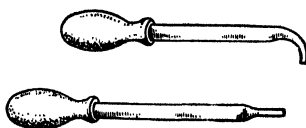


FIG. 24.—Pipettes, medicine-dropper type.

used. After standing several days, bacteria develop in these infusions in enormous numbers and soon there will be many protozoans, worms, and rotifers coming out of cysts and feeding upon the bacteria. Each type of infusion goes through a definite cycle, with the rise, maturity, and eventual decline of whole dynasties of interesting forms of life. Follow through these events, making slides daily, taking a census of the species present, and keeping records of each kind observed.

To mount specimens from any of these sources, one needs a pipette, a dipping tube, or both. By *pipette* (Fig. 24) in biology is meant the simple medicine dropper. Supply houses provide these and in addition a much larger type (Fig. 25) for reaching to the bottom of vessels and aquaria,



FIG. 25.—Large pipette for cultures in battery jars.

with a long tube and larger bulb. A *dipping tube* is merely a long glass tube—for instance, 1 ft. long—with the lower end drawn out over a flame to a tapered nozzle. Hold this tube in the right hand and submerge the nozzle to the point

from which the collection is wished, keeping the right forefinger over the upper end. This traps the contained air and prevents the entrance of water into the tube. When over the desired spot, raise the forefinger slightly for a moment,

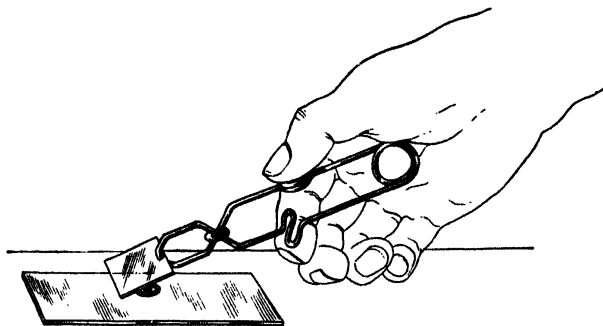


FIG. 26.—Mounting a cover glass with cover glass forceps.

whereupon the water rushes into the tube, carrying debris and organisms with it. Replace the finger and lift out the tube. Carry the tube across to a slide lying flat on the table before you, release the finger ever so slightly and thus allow one or two drops to fall to the center of the slide.

Samples should be taken, in the case of each culture or infusion, from the bottom, sides, middle, and top surfaces. Some organisms, like the famous amoeba, are bottom crawlers and will not be found elsewhere; others, as paramecium, are free swimming throughout all portions of the container; still others, as euglena, will collect in enormous numbers on the side facing the light.

One large drop is best, placed in the center of a slide lying flat on the table. Always include a bit of the debris along with the water, as around such material the organisms congregate to feed. Pick up a cover glass with a forceps and carry it to the slide. Allow the far edge of the cover to touch the slide, on a slant, beyond the drop of water (Fig. 26), then simply let go with the forceps and the cover will fall into place, the water spreading out into a thin film immediately. Some microscopists advise lowering the cover more

gently, often using a needle for this purpose (Fig. 27); others prefer holding the cover between thumb and forefinger, by the edges (Fig. 28) instead of with a forceps. In any case let one edge of the glass touch the slide first, then lower it



FIG. 27.—Mounting a cover glass by using a dissecting needle as a prop.

or let it fall into place. Mount the slide on the stage of the microscope, the covered portion over the aperture, and let it stand 1 or 2 min. for the inhabitants to recover from the shock of such rough handling; then proceed to study them.

The stage must be level for these observations. If your instrument has a permanently tilted stand, with no inclination joint, arrange to prop up the rear leg or portion of the base with a rubber eraser, block of wood, or other convenient object of correct height. Be careful in using the spring clips. If, when moving the slide about, one of these should come in contact with the water at the edge of the cover, some water will be drawn out over the slide and smear things up generally. Keep the clips far apart and away from the cover.

Fast moving protozoans may be slowed down, for detailed studies, by a variety of means. The following are

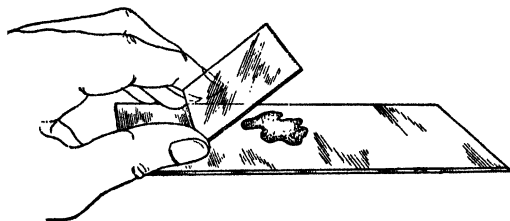


FIG. 28.—Mounting a cover glass with the fingers.

recommended: (1) add some cotton fibers, pulled out into a very thin mat, to the water on the slide before the cover glass is put on. This merely entangles and traps the animals but has the disadvantage of interfering with observation. (2)

Introduce a jelly of some sort into the culture drop so that the animals are mechanically impeded by the thickness of the solution, but not harmed. Various authors recommend gelatin, gum arabic, gum tragacanth, or quince-seed jelly, any of which may be used, but some experimentation will be necessary to find out the exact amount to add in order to secure the correct viscosity. Such forms as paramecium should retain their natural shape and be free to move slowly. (3) Add a poison that will eventually kill the inhabitants of the drop, but will do so slowly; meanwhile there will be 10 min. or so when the creatures are quieted down but still alive, and more or less normal in shape. After this period they become distorted and finally die and disintegrate. For this purpose, one drop of formalin in approximately 100 cc. of the culture is perhaps best and cheapest. Such narcotics as epsom salts, chloral hydrate, ether, and others have also been used, but are either expensive or difficult.

As a mounted drop stands, the water evaporates from under the cover glass in spite of the relatively minute surface exposed to air. A slide will last 20 to 30 min. on the average; toward the end of this time there is a considerable interval during which the cover is drawn so close to the slide that the animals are compressed and impeded. This is an excellent time for high power study of internal details, without resort to any other method at all, especially with the larger ciliates, like paramecium, but it must be kept in mind that the shape is unnatural.

Sometimes, then, one purposely allows a slide to dry out; more often the aim will be to prevent such drying out in order to prolong studies, especially if something unusual is going on. Every 15 or 20 min. the water must be renewed; this is done by adding fresh culture from the original source, placing the mouth of the pipette exactly at the edge where the slide meets the cover, and adding one small drop. The new water will contact the old and immediately flow under. If done carefully, no water should get upon the top of the

cover. Observe that this will set up rather strong currents and probably sweep away the particular specimen being studied. One must be prepared for this and do it between separate observations, or try to follow the currents and keep the specimen in view, or search for it again.

When still lengthier studies are in order, the cover may be sealed on with vaseline; if the sealing is complete and excludes all air, the slides will last for 1 or 2 days in this condition. For this purpose a *vaseline gun* is desirable—something like a miniature of the grease gun used at filling stations. Remove the rubber bulb from a pipette and fill the glass barrel with vaseline. Hold the barrel vertically and fill it from the bulb end by adding vaseline in small amounts with a toothpick. Tap the nozzle end frequently on the table top, causing the grease to settle, without air bubbles, until the barrel is completely filled. Punch a small hole in the rubber bulb and attach it to the barrel. When pressing out vaseline, keep the thumb over this hole; when releasing the squeezed bulb, allow air to fill it through the hole, otherwise the dropper will suck air into the vaseline through the nozzle. If preferred, fashion a plunger out of a glass rod, a match stick, or other material that will make a fairly tight fit, and push out the vaseline with this, dispensing with the bulb entirely.

Slides may be sealed after mounting, but it is easier to prepare a cover glass in advance by laying down a ring of vaseline all around the edge, then inverting this cover over the drop of culture to be sealed in. Circular covers are handiest for all sealed preparations.

The *capillary siphon* offers another method of renewing water lost by evaporation. Rig up a stand that will support a beaker or flat stender of the same water as is being used in the culture, and adjust so that this container is as close to the slide as possible for working comfort, and above the cover glass. Lead a piece of string or thread from the water to the preparation, the lower end passing just under an

edge of the cover glass. Water will pass by capillarity and gravity along the string and keep the supply renewed.

A better and more advanced procedure is to employ the *hanging-drop* technique, for which a depression slide is



FIG. 29.—Depression slide with hanging drop.

required (Fig. 29). Vaseline a cover glass as just described; then, in the center, place one small drop of the desired culture and invert this over the depression in the slide. Breathe into the depression first to moisten it. The size of the drop is the trick here—it should be as large as possible without making contact with any part of the slide, and hence remain as a hanging drop, attached to the cover glass only. Of course, it is entirely in order also to fill the depression completely, or to fill a deep-well slide (Fig. 18) and seal on the cover, thus making a miniature aquarium which can be studied for two or more days at leisure.

A word about contamination: for each culture bottle or infusion jar keep a separate pipette and either label it or indicate by matching colors the right pipette for each container. A spot of red paint on the bottle and its pipette; a red thread tied around each; both of them labeled I or A—there are many ways of keeping the pipettes separated. The second group would be identified by another color, number, or letter. It is highly important not to contaminate one culture with even a trace of another. As to water to be used in renewing that lost by evaporation, the best of all is water from the original source. Thus it is good practice to bring home a culture bottle from a pond that has proved rich in specimens, and also a pint or quart jar of extra water from the same site. When renewals must be made otherwise, the first choice is rain water, the second well water. If tap water must be used, allow a jarful to stand 1 or 2 days uncovered to exhaust the excess oxygen; then cover it and keep it as a reservoir of supply. Many city waters are chlorinated or treated chemically so as to be entirely unfit



for any use of this sort; they will kill many or all of the organisms you have collected.

**Making Up Solutions.**—In order to stain protozoa in temporary mounts and thus bring out the nuclei or other structures, two procedures have been used: lethal and vital dyeing. *Lethal* chemicals may stain, but at the same time they are poisonous and will kill the animals. Iodine solution is one of the best of these; methyl green and methyl violet are also excellent. *Vital* or *intra-vitam* stains color organisms or their parts but do not kill them, neutral red being one of the most widely used; Janus green, Bismarck brown, and methylen blue are other favorites.

*Iodine solution* is prepared by adding as many crystals of iodine to 70A (see abbreviations, page xi) as the alcohol will dissolve. Whenever a crystal or other solid is added to a liquid up to the point where no more will dissolve, the result is termed a *saturated solution*, as in this case. Make up 100 cc. of iodine solution and place in a labeled bottle to form the beginning of your collection of reagents for microscopy. To stain a slide of protozoan culture, add one very small drop of this solution at one edge of the cover, allowing it to run under. Some workers prefer to add the new reagent—whatever it may be in any case—at one side of the cover and at the same time, with a torn-off scrap of filter paper, draw out the old liquid from the opposite side, thus effecting a replacement of fluids without a corresponding increase in volume.

*Neutral red* has many uses, sometimes in strong solutions as a regular biological dye. But for intra-vitam work an exceedingly dilute mixture is made, as follows: make a 1% solution of the stain in distilled water, by which is meant 1 g. (gram) of the dry, powdered stain to 100 cc. of the solvent—in this case distilled water. This now becomes the *stock solution*, i.e., it provides a stock from which other mixtures can be made. It, too, should be bottled and labeled and kept for the future. One drop of this stock is now added to half

a tumblerful of pond, rain, or tap water, making a solution so very dilute that no color whatever is visible; yet this minute amount will stain protozoa without harming them. One drop of this final solution can be added to water on a

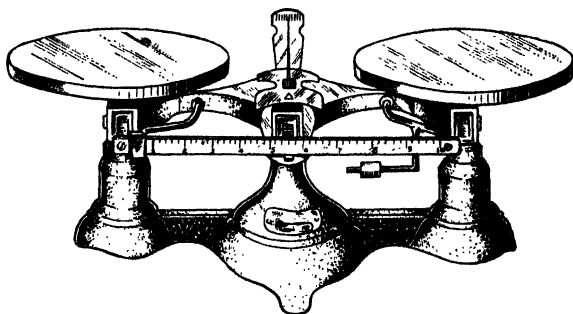


FIG. 30.—Chemical balance of a style suitable for the microscopist.

slide or, if you have extra cultures to spare, put one drop into the culture bottle, thus staining all the inhabitants directly.

The foregoing procedure now makes it necessary to explain how solutions in general are manufactured. You can buy many of them ready made or have them compounded by the corner druggist, but in so doing you will lose half the fun and experience as well as take on a much greater expense. The case is like photography as a hobby: no one ever makes much progress or becomes a serious fan until he develops and prints his own pictures. So the microscopist, to be worthy of the title, should early learn to make up nearly all of his own reagents. The outlay in dry stains, chemicals, and equipment may be greater at the start, but there is a decided saving over a period of time.

Two types of apparatus are needed: balances for weighing solids and graduated cylinders for measuring liquids. There are many sorts of *balances* or *scales* on the market, from simple to complex. Any kind that will weigh as small an amount as 1 g. with fair accuracy will be suitable for almost all of our purposes. Figure 30 shows one of the simpler and less expensive of the standard models. Recently there has

come to our notice the Raygram weigh-spoon, which we can recommend (Fig. 31). The chemical is placed in a spoon which is suspended at its center by a short chain, held by a finger. A sliding weight in the handle is moved to com-

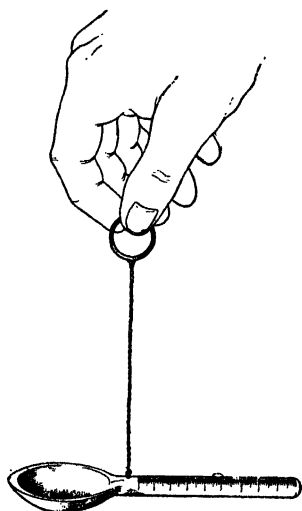


FIG. 31.—Raygram weigh-spoon.

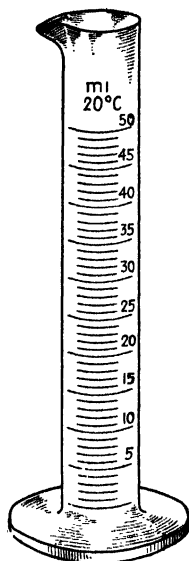


FIG. 32.—Graduated cylinder.

pensate for the amount added to the spoon, until the whole hangs perfectly level horizontally. This outfit is very inexpensive and is accurate from  $\frac{1}{2}$  to 10 g. in  $\frac{1}{2}$ -g. intervals. The gram is 0.032 oz., or 1 oz. weighs 31.1 g.

*Graduated cylinders* (Fig. 32) measure fluids in cubic centimeters (cc.), also termed milliliters (ml.), since there are 1,000 in a liter. One fluid ounce contains 29.57 cc., or 1 cc. equals 0.034 fluid ounce. These cylinders are obtainable from any supply house in several different sizes. For elementary work, one is sufficient. In this case get a medium size, as the 100-cc. capacity; if much work with them is to be done, buy three sizes, in capacities of 25, 100, and 500 cc.

Solutions are expressed in percentages of the solid, to be added to 100 cc. of the solvent (liquid); thus a 10% solution

of sodium chloride would be prepared by weighing out 10 g. of salt and dissolving them in 100 cc. of water. Unless otherwise specified, the solvent is always water—usually distilled water. This fluid was once somewhat of a problem for the individual not equipped with a still, but now it is readily obtainable everywhere since all garages and filling stations maintain a supply for batteries.

**Alcohols.**—Among the most widely used of all reagents in microscopy is *alcohol*; unless this word is qualified by some adjective, grain or ethyl alcohol is meant,  $C_2H_5OH$ . A product of fermentation, strong alcohol is prepared at distilleries by repeated fractional distillations and brought up to a maximum strength of 96 to 97%. The remaining small percentage of water cannot be removed save by treatment with some dehydrating agent as quicklime, forming *absolute alcohol*. This is taken to be 100% for all practical purposes and is so designated in this and other technique manuals. Actually there is always some trace of water remaining, and commercial absolute alcohol is about 99%.

*Rectified spirit* is the distiller's term for his purest alcohol, averaging around 96%. The U. S. Pharmacopoeia recognizes 91% as rectified spirit; this percentage differs in other countries so that the term has no exact meaning. *Proof spirit*, another distiller's phrase, indicates 50% alcohol by volume, 45.5% by weight, this being 100 proof. Hence a liquor advertised as 80 proof has 80% of a 50% alcoholic content, or in other words is a 40% alcohol.

Pure commercial grain alcohol varies between 95 and 97% strength, but as used in microscopy is always considered as 95. For exact determinations, an alcoholometer can be used. This instrument is like the ordinary hydrometer, and operates on the principle that different strengths of alcohol have different specific gravities. By reading on the scale at the level of the surface of the liquid, as the alcoholometer floats, the strength of the mixture may be known. Most of us are familiar with this operation when we have the water-

alcohol in our car radiators tested against freezing on cold winter mornings.

Colleges, hospitals, and such institutions can buy straight grain alcohol tax free by the barrel for scientific use, after complying with Government regulations; the individual microscopist must purchase his supply retail, in smaller quantities, and finds it fairly expensive. During national prohibition this reagent was impossible to obtain; since repeal, it may be had from any concern owning a liquor license, such as certain drugstores, and most liquor stores keep a small stock on hand. For the routine technique in microscopy, secure and use this alcohol if you can.

*Denatured alcohol* is ethyl alcohol rendered unpalatable or injurious for internal consumption by the addition of small amounts of some other substance, such as methyl alcohol or pyridine. Thus treated, such alcohols are freed from the internal revenue tax and made available for industrial or domestic use at a low cost. Rubbing alcohol for hospital and athletic massages is a familiar example. A number of standard formulas for denaturing are recognized, some of which make an alcohol suitable for microscopy, others not. If this sort of reagent is used, try to get Formula No. 1, the best of all for our purposes.

*Methyl or wood alcohol*,  $\text{CH}_3\text{OH}$ , is manufactured by the distillation of wood and other substances and is provided in about 90% strength. It is widely used in the arts as a solvent, in chemistry, and in alcohol lamps, and is fairly satisfactory in microscopy though by no means so good as ethyl alcohol. If used, it must be remembered that this substance is extremely poisonous. If taken internally, it will cause blindness, coma, and often death; externally, it is highly injurious to the skin, and the fumes are dangerous to the eyes. *Methylated spirit* is ethyl alcohol denatured with 10% methyl alcohol and is one of the better denaturing formulas for microscopy.

Other alcohols, such as butyl and propyl, are considered in Chapter 15.

**Dilutions of Alcohol.**—In connection with the iodine solution previously mentioned, we specified a 70% alcohol (designated in this manual as 70A) as the solvent. How does one go about making up a 70% solution from a 95% solution? Although it sounds difficult, in actual practice the procedure is very simple. Pour the 95A into the graduated cylinder up to the 70-cc. level, then add distilled water until the whole stands at the 95-cc. mark. From this one can see the general rule to apply: put in the 95A to the number of cubic centimeters represented by the strength wanted, then add distilled water to bring the whole to 95 cc.

**Examination of Fresh Blood.**—Blood, both human and animal, may be stained and permanently mounted, as will be described in Chapter 8, but it should also be examined in the fresh condition. Kill a frog by one of the methods advocated in Chapter 4 and obtain a large drop of blood; place this at once on a slide and immediately add a vaselined cover glass. The important factor here is speed, to get the blood covered before post-mortem changes, including clotting, can take place. The large, oval, nucleated cells of frog blood are very interesting in their fresh state, contrasting decidedly with the smaller, circular, non-nucleated disks of mammalian blood.

Human blood of course carries even more interest, with its profound significance in physiology, medicine, and criminology. In obtaining a drop for mounting, some care must be exercised to prevent subsequent infection. The preferred places for pricking the skin are the base of the thumbnail, the tip of a finger, or the lobe of an ear. Always wipe the part first with a bit of cloth or cotton saturated with 95A, then sterilize the needle to be used by passing it through a flame or by wiping it out of alcohol or iodine. After the drop of blood is obtained, sterilize the puncture by applying tincture of iodine.

Suppose the thumbnail is selected for this operation. First swing the arm violently to drive blood to its extremity,

then wrap a handkerchief or strip of gauze tightly around the thumb in a spiral, starting at the base. This causes the thumb to become swollen with blood and makes the obtaining of a number of samples easy. Prick the upper surface

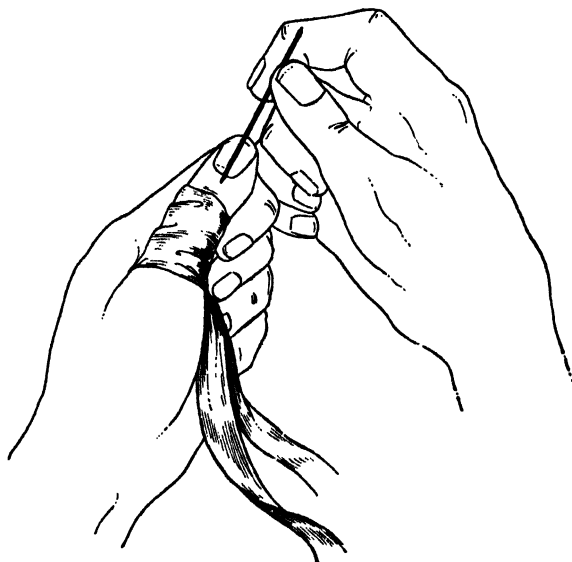


FIG. 33.—Technique of pricking finger to obtain drop of blood.

just back of the nail (Fig. 33), whereupon a large drop of blood will pop out. Mount and cover immediately as previously directed for frog blood. If the lobe of the ear is preferred, have someone else obtain the sample for you or use your confederate's ear.

**Circulation of the Blood.**—One of the most absorbing sights in microland is the actual circulation of blood as it courses through a network of vessels. Several animal forms have been commonly used for this observation; the web of a frog's foot, the tail web or gill filaments of tadpoles of both frogs and salamanders, and the gills of a clam. Figure 34 shows a suggested setup for the first of these, the frog anesthetized or pithed (Chapter 4). Wrap the animal in a

wet cloth so that the skin will not dry out but leave the nostrils exposed for breathing.

A sheet of cork is arranged with two legs as supports at one end, and an aperture to correspond with that of the

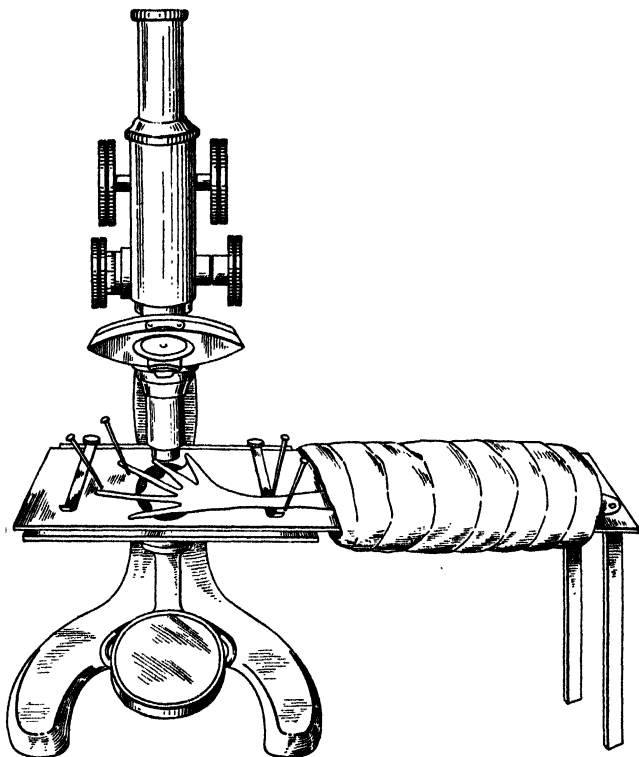


FIG. 34.—Setup for observing circulation of the blood in web of foot of living frog.

microscope stage. The frog is held to this sheet with a cheesecloth band or string and so positioned that a web between two of the hind toes can be stretched, not too tightly, over the aperture. Insert one or more pins, as needed, through the web, just inside the toes, to hold the web in place. Occasionally moisten the web with a drop of water. For high power it is best to use a broken bit of cover glass laid directly upon the web.



**Hair and Fur.**—Cut a few short lengths of hair from your head and from persons having hair of different color and texture; also obtain some from domestic animals and household fur garments. Two or three pieces  $\frac{1}{2}$  in. in length will suffice and do no harm to fur coats or neckpieces. Handle the specimens with forceps and keep them in separate, labeled glassine envelopes, homeopathic vials, or veterinary capsules. See that the slides and covers to be used are extra clean, as any dust is fatal to this work.

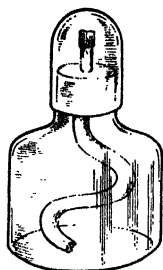


FIG. 35.—Alcohol lamp.

Grasping one or more hairs with the forceps, wave them about gently for a minute or more in a vessel containing a mixture of equal parts of 95A and ether, to remove all grease and dirt, then lay them on the slide and separate them with needles. Put on a cover glass and warm the slide over a low flame, as that from an alcohol lamp, until the hairs are perfectly dry. Place the slide on the microscope stage for examination, the stage level. Avoid drafts and do not breathe on the preparation which, not being sealed, is easily disturbed.

An *alcohol lamp* (Fig. 35) is a very essential addition to your outfit. Although inexpensive to purchase, one may be fashioned from an empty ink bottle. Soak off the label, then clean and dry the bottle. Select a cork of suitable size and bore a central hole through it to accommodate a short length ( $\frac{3}{4}$  to 1 in.) of brass tubing, as from a curtain rod, which should protrude slightly beyond both the top and the bottom of the cork, and be of  $\frac{1}{4}$ - to  $\frac{3}{8}$ -in. diameter according to the size of wick procurable from your local ten-cent store. Allow about  $\frac{1}{4}$  in. of wick to project above the cork and fill the bottle with full strength commercial alcohol, either ethyl or methyl. If a cap of some sort can be made to fit over the lamp when not in use, loss of alcohol from evaporation through the wick will be diminished.

**To Demonstrate Miscibility of Fluids.**—Miscibility, a word now obsolete in common usage, is still retained in the vocabulary of science to mean mixability and is introduced here at this time because of its importance in processing permanently mounted slides, as will be seen in the following chapters. Two substances are *miscible* when they will mix completely, as alcohol and water. Place a clean slide on the microscope stage and on this put a large drop of water. Focus on this drop with the light cut down. Now, while looking through the microscope, add a drop of 95A to the water; or add the drop and immediately observe the results. A cover glass is not necessary but may be used if desired. Alcohol has a strong affinity, as the chemist says, for water, and mixes with it so rapidly that violent *convection currents* are set up. If, instead of these two extremes, you mix drops of water and 35A, or of 70 and 82A, no such vigorous currents will form, the mixing being more quietly effected. This experiment shows why a *graded series of alcohols* is used in microscopy. Whenever it is necessary to transfer objects from water to 95A, it is almost always done by passing through a series consisting of 35, 50, 70, and 82% strengths of alcohol, thus providing intermediate steps which prevent damage by convection currents.

Now similarly mix a drop of water with one of either xylene or turpentine on a slide and examine. Note that a milky fluid results; these substances are not miscible. In fact, either xylene or turpentine may be used as a test for the presence of water since they will not tolerate even a slight trace. To show this, mix one of these with 95A, which you recall contains 4 to 5% water, and observe the results. Many other similar experiments in miscibility may be performed.

**To Observe Crystallization.**—The formation of crystals (Fig. 36) is readily seen under low magnification and provides a most fascinating field of study. Any of a great many chemicals may be used, such as common salt, and the

method is simplicity itself. Dissolve as much salt as will go into solution (saturated solution) in a small amount of hot water. As this cools, the solution becomes supersaturated and throws down its excess load, since cold liquids will not



FIG. 36.—Hydroquinone crystals (40 X).

dissolve or hold in solution as much of a salt as will heated ones. Place one large drop of the hot salt solution on a slide and watch under the microscope to see cubes crystallize out.

Still more interesting are such chemicals as potassium permanganate, potassium bichromate, and hydroquinone, the last being commonly employed in photographic developers and forming very elaborate and beautiful crystals.

**To Observe Brownian Movement.**—Brownian movement, or pedesis, is a phenomenon of all extremely fine

particles of matter when floating in a gas or fluid, and bears the name of Robert Brown who first demonstrated it in 1827. The movements consist of dancing, irregular, but continuous motion, haphazardly in all directions, and are fascinating to watch. Any of a number of substances that can be finely divided may be used, as ground-up carmine (rouge), lampblack, chalk dust, starch, powdered aluminum, pumice stone, and the like. Mount a bit of one of these in a drop of water. Gage states that the action is accelerated by adding small amounts of either gum arabic solution, silicate of soda, or soap to the water, and that sulphuric acid and various saline compounds exert a retarding influence or check the motion altogether.

Use the high power, dry lens; see that the stand is vertical; and arrange a bright light with the diaphragm cut down. Various inert larger masses will appear in the field and only the finest particles will exhibit the movements. Striking observations of pedesis may be made also with dark-field illumination and with polarized light. Some supply houses provide a smoke chamber to be mounted on the microscope stage, filled with cigarette smoke, and then strongly illuminated through a small aperture in the side, revealing the amazing Brownian movement of carbon particles in air—a sight never to be forgotten.

**Other Objects.**—The list given thus far in this chapter is far from complete. A great many other substances may be mounted for temporary examination and the reader is urged to browse around and try out all manner of objects, mounted dry or in a drop of water. Among additional suggestions are the following:

*Household Objects:* dust from floors where rugs are present and from the attic, cellar, or other rooms where no rugs are placed; cosmetics, dentifrices, and drugs of various sorts, powdered or scraped; foods from the kitchen, including scrapings from potato, rice, wheat, etc. for starch grains, and using the iodine solution as a test since it will turn

starch blue; ground coffee, shredded tea leaves, pulverized cereals, spices, pepper, salt; and additional paper, textiles, hairs, or furs.

*Organic Objects:* infusions of many kinds other than those already given are possible, and the opportunities for field collecting of protozoa, algae, and other minute animals and plants is limitless. Feathers from birds, scales from reptiles and fish, wing scales from butterflies and moths scraped off onto a slide and mounted dry, pollen grains, seeds and spores of plants, stamens, moss capsules and leaves from mosses and ferns—all these offer many hours of entertainment and instruction with your microscope.

*Inorganic Objects:* an almost endless series of mineral and chemical crystals is available. Try many forms of sands and soils; examine asbestos, steel wool, wire gauze, gem stones, and jewelry settings; inspect paper money, coins, stamps, steel engravings, and other types of printed reproductions. Some of these are best by transmitted, others by reflected light.

## CHAPTER 3

### SIMPLE BALSAM MOUNTS

*In This Chapter:* balsam, xylene; permanent mounts of paper, half tones, textiles; slide drier; collecting insects; mounting fly wing, soils, fingerprints, crystals; cover glass supports; cleaning and labeling finished slides.

**T**EMPORARY mounts serve their purpose. In most cases, however, we shall wish to have them permanent and stored in a slide collection so that they can be taken out and examined whenever we choose. For this to be possible, the object must be sealed in either a fluid or a solid medium, termed a *mountant*, and of the many such substances employed in microscopy, none is more important or more widely used than balsam.

**Canada Balsam.**—There are a number of balsams, prepared from the resins of various trees, but when the term is used without any qualifying adjective, it always designates canada balsam. This is a turpentine derived from the tree of the same name, also called the balsam fir, fir balsam, fir pine, and balm of Gilead—*Abies balsamea*, a native of canada and the northern United States.

The bark of this fir is smooth but covered with numerous vesicles containing the exuding resin. The balsam of commerce is collected by breaking these as well as by incising the bark. The substance thus obtained is a clear, light-yellow, viscous, aromatic resin, liquid at first but gradually hardening to a transparent solid on exposure to air and the evaporation of lighter ingredients. As collected, this material is known as *natural balsam* and is often used for slide mounting in this form. More commonly it is evaporated to a solid, then dissolved in xylene, and marketed as *xylol*

*balsam*. During such preparation it is customarily filtered through paper and the term "paper filtered" in supply house catalogs indicates such treatment.

Finally, as to terminology, most balsam is slightly acid in reaction, often an undesirable feature in microscopy. When this acidity is corrected, the product is advertised as *neutral balsam*. One can neutralize his own balsam and maintain it so by adding a pinch of pure sodium carbonate or by keeping two or three small pieces of marble in the *balsam bottle*. Every microscopist should provide himself with this item of glassware (Fig. 37), which is low and squat to prevent overturning and has a glass cap and applicator rod.

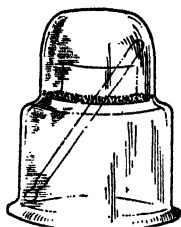


FIG. 37.—Balsam bottle.

Thickness is an important consideration with balsam. *To thicken*, expose it to air in a warm place free from dust, as in the evaporator or slide drier discussed later in this chapter. *To thin*, add xylene and stir until dissolved. Balsam that is too thick makes a gummy, dark-colored mount on which adjustment of the cover glass may be difficult; if too thin, air bubbles will work in under the cover and replace the xylene as this oil evaporates. A little experimenting will be necessary to get the average desired viscosity. For most purposes, the balsam should flow readily from the end of the applicator rod in a thick, ropy stream and, at the end of such flow, deliver two or three separate drops; these drops are used in slide mounts. One large or two small drops are right for most mounts; less for very thin objects, such as thin sections of small area; more for very thick objects, such as a whole insect.

Because of the fact that it may be applied as a liquid but will later harden to a solid, because of its perfect transparency, and because of its high refractive index (1.52), which is the same as or but slightly less than that of glass, canada balsam constitutes an ideal mountant, generally preferred for most purposes. It is also used in photography,

as an ingredient in varnishes, and as a cement in the optical industry, especially in connecting the separate lenses in building microscope objectives.

Damar, camsal balsam, colophonium, and sandarac are other resins, each with enthusiasts who use them, but they are generally considered inferior to canada balsam.

If materials are absolutely dry and contain no water in any sort of combination, as is true of a piece of paper, fabric, pinch of sand, or thoroughly dried insect wing or plant seed, they may be mounted on a slide directly in a drop of balsam, without any preliminary treatment. In almost every case, however, since xylene is the solvent for balsam and is an essential oil that will render specimens translucent, it is best practice to pass objects through xylene first.

**Xylene.**—This oil is generally considered the most satisfactory of all *clearing agents* in routine microscopy. Clearers are so called because they impart transparency to objects. The name “xylene” comes from the Greek for wood; the ending *-ene*, as seen also in benzene and toluene, is the English suffix for chemicals of the hydrocarbon series; *-ol* is the German form, as xylol, benzol, and toluol. One sees either the form “xylene” or “xylol” used more or less indiscriminately in recent publications; they are synonymous.

Xylene is a colorless, oily liquid, rough on the hands and with a characteristic pungent odor. It is a hydrocarbon of the benzene series, with the formula  $C_8H_{10}$ , and is itself the parent of a whole series of other compounds. It may be prepared from coal and wood tar, certain kinds of petroleum, and also synthetically. It has the property of hardening tissues—sometimes an advantage, sometimes otherwise—and evaporates so rapidly that one must use care not to allow material taken from it to dry out. It is miscible with alcohol (100A), melted paraffin, and balsam, being, as we have seen, the commonly employed solvent for balsam; it is not at all miscible with water. We have already noted that



a small trace of water is sufficient to turn xylene milky, so that it can be used as a test for the purity of 100A.

The length of time for soaking objects in xylene varies with their size, thickness, and porosity; the proper time can be told by inspection—objects not fully cleared have some remains of opaqueness in their appearance, whereas fully cleared materials look entirely translucent. In general 10 min. is safe for most subjects; 5 min. is often ample. Various items of glassware are suitable as containers, the small stenders being most handy and conserving of the xylene. In clearing such waterless whole objects as paper and textiles, the same dish of xylene may be used repeatedly, whereas with organic materials, such as insect parts, it should be employed only three or four times and then discarded. Always keep the dish covered, to prevent evaporation and to exclude dust and moisture.

**Making a Permanent Slide of Paper.**—Clip a piece of newspaper, including a portion on which printed letters appear, to about  $\frac{1}{2}$  in. square, and picking it up with forceps, drop it into a small dish of xylene for 10 min. Lift it out with forceps and allow it to drain over the edge of the dish until excess xylene has run off; then transfer it to the center of a blank slide and watch while the xylene evaporates, which occurs rapidly. When the first faint haze appears on the surface, put on one fair-sized drop of balsam, then add a cover glass in the same manner followed in mounting a drop of pond water.

If it is allowed to stand too long before adding balsam, too much evaporation will occur and the preparation may appear dull or even opaque when finished. It is better to add the balsam too soon rather than too late, as any excess xylene will evaporate from under the cover anyway. A bit of practice will make this a simple matter, as also the correct amount of balsam to use. When the cover falls into place, the balsam should spread out to fill all of the space under the cover, but no more; it should not run out onto

the slide from under the cover. Some workers recommend pressing gently on the cover with a blunt implement, such as the eraser end of a pencil or the butt of a needle handle, but this is not necessary with so thin and flat a mount as a piece of paper.

Excess balsam can be cleaned up, as explained later. If insufficient balsam has been used, more may be added by the same technique used in adding more pond-water culture to renew a temporary water mount, that is, by applying a small quantity exactly at the edge of the slide and cover to contact the balsam already present. Air bubbles under the cover will generally work out by themselves; if they do not, they may be pricked with a heated needle, run under the cover.

With the slide made, the next task is to dry it, since the balsam is fluid and will run. Hence the slide must be kept perfectly flat until completely dry and hard. It may be desirable or necessary to examine certain slides while still fresh; in such instances have the stage of the microscope flat and keep the spring clips away. It is easy to foul them with balsam and ruin the preparation unless one is careful. A sure way is to take them off and lay them aside during such an inspection, or swing them back toward the rear.

**Slide Drier.**—Slides dry by evaporation of the xylene in the balsam and most mounts will harden in a week. Very often it is preferred to have them ready for use sooner, and a *slide drier* is therefore one of the more useful accessories. For this purpose any form of gentle heat will serve. In the winter, when the house is heated, a flat tray of wood, veneer, plywood, or composition board, or tin may be fixed up to stand above a radiator or register—not resting directly upon the hot radiator but supported by legs or hung above it. However, a year-round model is more practical and dependable and may take the form of a box about 1 ft. cube or a trifle larger, with one or two shelves for the slides, preferably resting merely on cleats so that they can be

pulled in and out. Trays are best made of heavy, coarse-meshed ( $\frac{1}{4}$  to  $\frac{1}{2}$  in.) galvanized screening, with edges crimped or bound. Some holes are arranged around the top of the box sides for ventilation and a bulb of not more

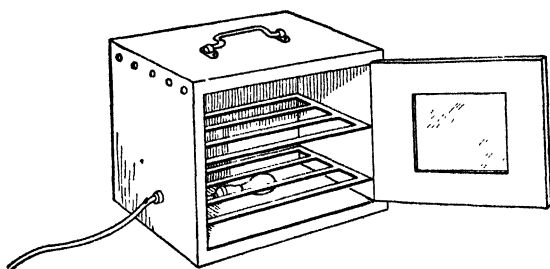


FIG. 38.—Gas-stove oven converted into a slide drier.

than 40 watts placed inside to provide the heat. Take care that the bulb does not rest on wood, which will char or even catch fire. Have it suspended near the bottom or resting on a small, thick sheet of asbestos.

One of the best forms of slide drier for the amateur is made from a gas-stove oven (Fig. 38) at small expense. Secure one of these cubical metal boxes, new or secondhand, designed to be placed on top of a gas burner for stoves that have no built-in oven. There is a hinged front door with glass for observation, and two adjustable shelves within made of bars, to allow heat to circulate; ventilating holes are also provided. All that one needs to do is to drill the rear side near the bottom to receive a porcelain or metal tube through which passes the electric cord, and support a light socket inside near the bottom center, not quite touching the bottom. A stone slab or sheet of asbestos had best be placed under the oven, especially if it stands on a wooden table. Such a device may be run day and night continuously whenever slides are to be dried; most mounts will be ready within two days.

Interesting papers to prepare in order to have a comparative study collection are newsprint, bond, linen or rag tissue, cigarette, and wrapping papers. Another and

most interesting excursion in microscopy is to study modern commercial methods of reproducing illustrations. Clip portions of a line drawing found in a magazine and make a permanent slide to show features of a zinc etching; then compare it with a half tone (photographic) illustration, in which the use of a half-tone screen will be clearly seen under even very low magnification. Newspaper photographs are reproduced by means of coarse screens; the better ones on heavily calendered paper, in the more expensive magazines, use much finer screens. Again, the mechanics of the three- or four-color process, the Ben Day, and still other methods make slides that are fascinating to study. Mount a bit of the face of the girl on the magazine cover, and you will find that she is nothing but a jumble of colored dots. Reflected light is best for this work.

**A Composite Textile Mount.**—Ordinary slides of textiles of all sorts and of either fibers, threads, yarns, or fabrics may be prepared by the same method as for paper—simply clearing in xylene and mounting in balsam. An excellent preparation to show all parts in a single slide, hence termed a *composite mount*, is to clip a  $\frac{1}{2}$ -in. square from a piece of goods, such as cotton fabric, then with needles tease away several of the threads crossing one side, leaving the lengthwise threads protruding a short distance. This will show the fabric as a whole, with the method of weaving, and also separate threads with their twisted fibers. Now tease and fray out two of these threads to reveal the individual fibers and process the whole piece, mounting it in balsam. The slide will show fabric, threads, and fibers, all in one mount. Prepare such a slide for real silk, wool, cotton, linen, and several of the rayons. Still other interesting mounts in this group include mixed goods, as cotton and linen, cotton and wool, or wool and rayon.

**Collecting Insects.**—The microscopist in most cases will have much to do with insects and so should early learn some

of the methods and places for the collecting of these tiny but abundant forms of animal life. A number of detailed references are given in Appendix *B* which should be consulted, if possible, to supplement the brief instructions that now follow.

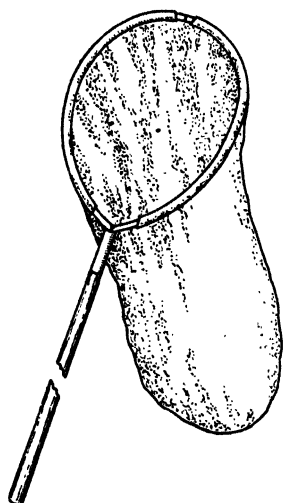


FIG. 39.—Insect collecting net.

An *insect net* (Fig. 39) is one of the main requirements. Some commercial nets are now so inexpensive that it hardly pays to make your own. However, the method is simple and can be outlined briefly. For a stick, use a 3-ft. length of bamboo or cane or a miniature (fireplace) broom handle. Plane or shave down two opposite flat surfaces at one end—the smaller end if there is a difference—for a distance of 2 in. Cut a 2-in. length of brass tubing of a diameter such that it will just slip over not less than 4 in. of this end of the stick with little clearance to spare.

For the frame, use galvanized or copper wire stout enough to resist bending when in use, and form a circle 12 to 15 in. in diameter. Sometimes you can find a pillar, barrel, or other cylindrical object of correct size around which to shape the wire. Leave two ends, one 2 in., the other  $1\frac{1}{2}$  in. long, which project away at right angles from the loop, and parallel with each other. Crimp the ends of these short pieces inward and fit them to the flat sides of the stick end, boring holes in the stick of slight depth to take the crimped wire ends (Fig. 40). These ends should now stand above the general level of the stick so that, when the metal sleeve is pushed forward over them, the fit will be tight enough to hold the frame rigidly in place, yet allow for instant detaching merely by sliding the sleeve down on the stick again. This type of frame has the advantage of being interchange-

able with others carrying nets of different mesh or size, and can be knocked down for transportation.

Another form, somewhat easier to make but not so flexible in use, is constructed by cutting a 4-in. length of metal sleeve of a diameter such that it can be hammered over the smaller end of the stick and fit very tightly. Drive 2 in. of this sleeve onto the stick and leave 2 in. projecting. Place the frame ends inside this hollow and fill with solder. A third method is to use flat spring wire for the frame and secure the two ends placed flat against the stick end by winding them tightly with wire, then binding with tape.

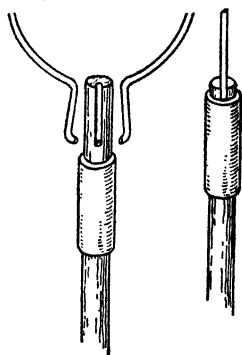


FIG. 40.—Method of fastening wire loop construction of insect net.

The net may be of several materials. If wanted for collecting insects in perfect condition to be used as dry, spread mounts, as in the usual pinned collection, select scrim for coarse work, bruxelles for a fine-meshed delicate bag. If to be used solely for catching insects destined for slide work, any cheap and coarse netting will serve. After a conical bag has been shaped, with a 2-ft. depth, sew the rim to a heavier strip of canvas. This border is doubled, forming a hem to receive the wire frame, and the two ends are left open to permit slipping the net on and off the frame for laundering or replacements.

Use fine nets for aerial captures only and exercise care not to snarl them on brambles or other objects. For aquatic collecting make or buy a heavier duty net, or construct one of wire screening. A very handy field item for aquatic insects and other animals is the *screen box* (Fig. 41), which is simply a wooden box about 24 by 12 by 9 in., with neither top nor bottom and with two slots cut in the ends as hand grips. Cover the bottom with rustproof screening and bind this on with small half round or molding. When worked through shallow ponds, by pulling this box toward you

among aquatic plants and raking up the bottom mud or sand, an amazing variety of captures is made with ease. Excess water drains off through the screening and, if the bottom is muddy, the contents may be cleared for examina-

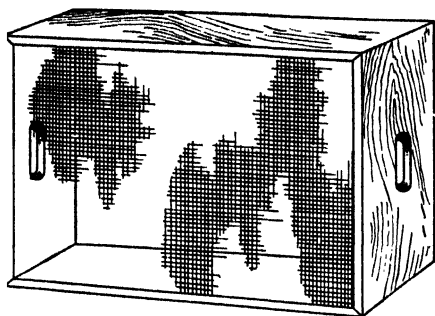


FIG. 41.—Screen box for aquatic collecting.

tion by jouncing the box up and down several times on the surface of the water to strain out the mud.

Specimens are transferred from the box or a water net to a *crystallizing dish* (Fig. 42), which is a shallow, cylindrical piece of glassware made in many sizes, excellent to carry afield for temporary examination of the visible specimens in a small amount of clear water and to isolate important finds from the general mess of trash in the box.

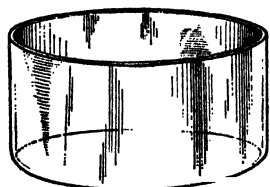


FIG. 42.—Crystallizing dish.

Collecting with the air net is not so simple as the funny papers would indicate, but is an art requiring practice and experience. The big butterflies and moths, fast-flying beetles, flies of many sorts, and dragonflies are not to be taken without skillful stalking and bagging; they are alert and agile. Wherever possible, swing against the wind so as to keep the bag bellied open. Swing hard, but watch out for snags around bushes; develop both a forehand and a backhand and alternate them rapidly, twisting the wrist at each turn and keeping the bag open. When a capture is made, instantly twist the wrist so that the bag

drapes over the frame and closes the opening. If insects have alighted on the ground or on a plant, bring the net down upon them smartly from above, then hold up the tip of the bag with the fingers as most species will fly upward and are then trapped in the narrow part of the net.

In transferring animals from the bag to the collecting bottle, work the captives into the small end of the net, then close the net in on them with the fist from below. Work the bottle up into the bag and peel the latter down over the rim of the bottle so that escape is prevented and eventually the captive is forced into the bottle. While the net is still across the opening of the jar, put on the lid or cork until the insect is stunned (cyanide bottle) or immersed (alcohol bottle); then remove the cork, bring the bottle outside of the bag, and replace the cork. If entire insects are wanted for pinning in a dry collection, the cyanide jar is best; for microscopy a wide-mouthed collecting bottle (Fig. 21) half filled with 70A

is sufficient, safer, and saves time. Waste alcohol from various techniques to be described later is excellent for this purpose and conserves funds. This fluid kills and initiates fixing (preserving) so that several of the steps from the live animal to the permanent slide are thus accomplished at one stroke in the field.

A fairly large straight-sided jar is generally preferred as a *cyanide killing bottle* (Fig. 43), or one of the newer non-breakable celluloid tubes. Unless you are experienced in chemistry, you should not attempt to make your own killing jar, since handling cyanide is flirting with the undertaker. Rather, we suggest that you purchase a ready-to-use charged jar from a supply house, or have one made at a drugstore. Keep the jar tightly closed at all times, save when adding or removing specimens.

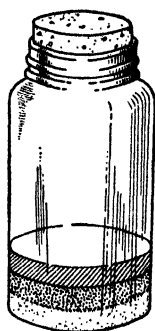


FIG. 43.—  
Cyanide collecting jar. Layers shown, from bottom toward top are: cyanide, sawdust, plaster of Paris.



In a great many cases, no net at all is needed in collecting insects and their relatives. *Hand-picking* is not only possible but can be practiced to a much greater extent than most people would think. Use a collecting bottle half filled with alcohol or a larger wide-mouthed jar equipped with a metal lid that screws on with a single short twist (Amerseal type), such as a pint mayonnaise bottle. Approach a bee that is busily occupied in gathering nectar from some plant and bring the bottle up from below, the lid down from above, and simply enclose the insect between the two. This is done gently and not too rapidly and is perfectly safe with most stinging insects as well as effective with many bugs, beetles, and flies. Still others may be picked from flowers by the fingers and dropped into alcohol. Certain bugs and beetles have the habit of dropping to the ground when disturbed on a plant; if the bottle is held just beneath them, they thus assist in their own capture.

Plants especially suitable for this hand-picking include all of the garden flowers, most vegetables, and goldenrod, Queen Anne's lace, milkweed, butterfly weed, sweet clover, willow catkins, and others whose odors attract a great many insects. *Beating* small trees and shrubs with a club will jar off a large assortment, and *sweeping* a net vigorously back and forth through tall grass will likewise produce a goodly crew, mostly of the very small forms preferred by the microscopist in making whole mounts. *Poisoning* and *trapping* are till other methods.

Another prolific source is an invasion of the *cryptozoic habitat*, by which is meant the home of many kinds of animals that remain concealed and go abroad little if any. On field trips, turn over flat stones, logs, bark, piles of leaves, and especially such trash of human manufacture as old pieces of tin, tar paper and roofing, old board signs, and similar objects. Here will be found a surprising array of animal life, varying from such larger creatures as field mice, moles, shrews, snakes, salamanders, and bumblebee nests to a great host of sow bugs, millipedes, centipedes, spiders,

roaches, termites, beetles, and ants and, in suitable sections of the country, scorpions, tarantulas, and other spectacular or venomous forms. Some of these will scurry away rapidly and it may require speed and dexterity to capture them. Aside from the larger spiders, scorpions, and centipedes, these animals are harmless and may be picked up in the fingers; the only very dangerous inhabitant is the black widow, a medium-sized, almost spherical, shiny black spider with a red hourglass-shaped marking on the under side. Scooping up in a bottle, trapping between two bottles, picking up with forceps, and still other methods will serve when in doubt. Certain interesting beetles are to be found beneath carcasses and cow dung in meadows.

*External parasites* include many mites, ticks, lice, flies, and fleas that may be gathered by searching the hair and hide or feathers of domestic and farm mammals and birds, as well as the chance opportunity afforded with wild animals shot in hunting. Remove them with the fingers or forceps and drop them into an alcohol bottle. Use a separate bottle for each species of host animal, as it is important to know the source of these pests.

Collect not only adult insects and their relatives, but also other stages in their life cycles, including eggs, nymphs or larvae, pupae, and pupal cases. A good manual of entomology is essential in studying the nature, occurrence, and seasonal distribution of these as well as adults.

**Mounting an Insect Wing.**—Perhaps the best choice of an insect part for preliminary experiments in making a balsam mount is a wing, preferably one of the larger species such as the blowfly, also called “bluebottle” or “greenbottle,” though the ordinary housefly is good too. Kill a fly in a cyanide jar or by cutting off the head; then with a forceps grasp a wing by the base and remove it, seeing that you get the whole wing without any mutilations. Put this in a pill-box to dry for 24 hr.; then remove with the forceps and place it on the center of a clean slide, the wing horizontal. Add

xylene for 1 or 2 min., then balsam, and a cover glass. Many other insect wings may be mounted in this same manner, though a better procedure is to dehydrate them before clearing, as explained in the two following chapters.

**A Simple Mount of Soil.**—Secure a sample of soil of some known type, such as clay, sand, loam, or peat, and place a teaspoonful in a pint bottle two-thirds filled with tap water. Shake and stir until all the particles are well broken up and separated; then, before the soil has a chance to settle, take a pipetteful and place two drops in the center of a slide. Store flat in an evaporator for 24 hr. to dry thoroughly. The water evaporates and leaves the soil as a thin film or deposit on the slide. Add a drop of balsam and a cover. The finished slide should show particles sufficiently dispersed that they may be studied without difficulty by transmitted light. If the soil is clumped into larger masses or particles are so close as to form a practically continuous sheet, the slide is not successful; the original mixture was too thick. Use less soil or more water.

By *evaporator* we wish to indicate briefly, without a repetition of words each time, some dry situation, entirely free from dust and dirt, where objects or slides may be stored in safety for short periods while they are drying out. A drawer in a desk or bureau, a small cupboard or cabinet, a large metal or wooden box, an incubator or oven not otherwise in use—any of these or other similar places will serve. It is quite necessary that the evaporator be of such close fit or in such a location that no dust at all can get upon the exposed slides.

**Mounting a Fingerprint.**—Although fingerprints as used in criminology or personal identification are customarily made on white cards for filing purposes, it is quite possible to make one directly on a slide, and an interesting series of different types may be permanently preserved in this fashion. The slide must be extraordinarily clean and free

from the slightest traces of grease. Use the glass-cleaning mixture and make the following two tests on one of the batch of slides so cleaned: (1) Breathe on the slide. The moisture of the breath should form evenly all over and disappear in the same manner. (2) With a fine-pointed brush, draw a line of water along the slide. It should take evenly throughout the stroke and should not run. Reject any slides that cannot meet these tests.

To make a fingerprint, use regular police methods, which require a very flat and smooth slab, such as a piece of marble, slate, plate glass, or porcelain, and on this place a small quantity of black printer's ink. Roll the ink with a rubber roller, which can be one of the type used by photographers in rolling prints or an old, cleaned typewriter platen, or a length of broomstick or similar wooden handle upon which has been pulled a shorter length of rubber hose. Roll the ink out into a perfectly smooth and very thin coating, then apply one finger in rotary fashion, laying the tip of the left side of the finger upon the inked slab and rolling the finger over to the right side, then lifting away cleanly. The pressure should be moderately firm, to ink all ridges on the finger, but not so forcible as to produce blurring. Now transfer the inked finger to the center of the slide and repeat the same operation, rolling the finger over and lifting it away cleanly. Repeat until a print is obtained that is uniformly heavy and clear all over, without smudges. It is preferable to have someone else grasp your finger and manipulate it for you, as a police official does in taking fingerprints. The slide is put away in the evaporator for several days, until absolutely dry; then balsam and a cover glass are applied.

It is also perfectly possible to make a fingerprint on a piece of thin tissue paper and then mount the paper for use with transmitted light; or on thin cardboard for inspection by reflected light, making permanent slides according to the method for paper, but omitting the xylene.

**Permanent Slides of Chemical Crystals.**—Prepare saturated solutions of various chemicals, as outlined in the preceding chapter, and in each case place a drop on a slide and dry in the evaporator. Most crystals may then be mounted in balsam but some are either dissolved or distorted by the xylene or the balsam and must be mounted in castor oil or glycerin. Such slides require rings or cells and the cover sealed on as in the methods explained in Chapter 6.

If not soluble in alcohol, saturated solutions may be dropped into this reagent, whereupon crystals will be thrown down at once and may be recovered with a pipette, placed on a cover glass or slide to dry, with or without gentle heat, and then mounted in balsam. Still other workers use the fusion method. Heat the salt on a slide until it melts, then spread the fluid with a hot needle and allow it to cool, when crystals will form. Add balsam and a cover glass.

Some crystals, as those of *santonin*, will assume different forms according to the temperature used with them. Dissolve them in chloroform, drop a bit of this solution on a slide to evaporate, and ring in castor oil. *Hippuric acid* is a classic for beauty of crystal form and is prepared by saturating in 100A, warming and placing a drop on a slide, then mounting in natural balsam or castor oil. Variations of form occur according to the atmosphere in which the crystallization takes place. Try a moist air, a dry air, and vapors of benzene, ammonia, and other reagents. The results will be surprising.

Still other crystals are secured by causing chemical reactions to take place in vials or on the slide. Thus calcium sulphate needles are produced when drops of calcium chloride and zinc sulphate are mixed and evaporated. Opportunity for experimentation in this field is well-nigh limitless.

In general, any materials that are perfectly dry and will not alter through decay may be mounted directly in balsam. Unless thin enough to pass light they cannot, of course, be

studied by transmitted light; we shall take up certain opaque objects later on.

**Cover Glass Supports.**—When objects of any considerable thickness are to be mounted, such as a hydra, worm,

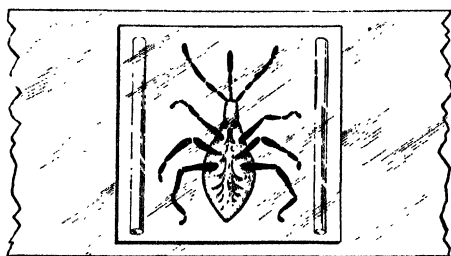


FIG. 44.—Whole mount of small insect, showing use of cover glass props.

or insect, it is best practice to prop up the cover glass around the edges to the same extent as it is raised in the center by the specimen, protecting both glass and object against crushing. For very thin supports use horsehair or broken bits of a cover glass, the latter having the advantage of being practically invisible when in balsam. Save the pieces when you accidentally break a cover and store them in a small jar or box labeled for the purpose. Use three equidistant props with circular covers—the familiar principle of the tripod; four with square covers, one at each corner.

In another container keep bits of a broken slide or strips cut across a slide with a glass cutter, or short sections of fine glass rod, to be used as supports for thicker materials. Figure 44 shows part of a slide on which an entire bug has been mounted, with the cover glass held up at the two sides by means of a very small glass rod. Such a mount requires more balsam than usual to fill up the extra space, and must dry for a correspondingly longer period. Still thicker preparations call for the making of a cell, as explained in Chapter 6.

**Temporary Labels.**—As soon as a stage is reached in any microscopical technique when an object is permanently fastened in some way to a slide, a temporary label should be attached. Whole mounts are labeled after putting on balsam and a cover glass; sections as soon as they have been cut and affixed. Since the purpose is to avoid all possible errors in the final label, this step should not be neglected. When one is carrying through several or many slides at once, as he will frequently do after passing the beginning stages, it is very easy to forget which slide is which unless they are labeled.

Tear up any form of gummed label into small pieces. Paste one of these on one end of the slide being processed and write merely a serial number on it, tying up with the same number on a record card or in a notebook. Or use one of the following:

*A glass-writing pencil* is one made of a soft wax and comes in several different colors. Fine writing is not possible, but letters or numbers are easily made and are rubbed off with a towel when the need for them has passed. We prefer the yellow pencil for microscopy.

*Carborundum points*, as supplied for the engraving trade, are very inexpensive and will scratch glass so that numbers, letters, or even complete data may be written directly upon one or both ends of the slide, with the advantage that there is no label to become torn, soiled, or washed off. *Diamond points* or pencils are likewise available but are much more expensive.

*Glass-writing ink* can be manufactured according to a number of formulas given in chemistry manuals. One of these is the Diamond Ink of Eimer and Amend, another is the Gold Seal permanent laboratory inks of Clay-Adams, available in black, white, red, and blue.

**Cleaning Finished Slides.**—When a balsam mount is completely dry and hard, it is ready for a cleaning and polishing. Soak the tip of the slide bearing the temporary

label in water to remove it, or wipe off the wax of a glass-writing pencil, if either of these methods was used. Then comes attention to any excess balsam that may have exuded from under the margin of the cover. The bulk of this is best scraped away carefully with a small knife blade, the balsam flaking off easily. Be careful not to touch the edge of the cover itself with the blade; it is very easy to knick and shiver this fragile bit of glass and ruin the mount. Scrape right up to the cover without touching it. A little practice and one or two mishaps make one expert at this very quickly.

A small bit of rag wrapped around a toothpick or matchstick and moistened with xylene makes an excellent swab for finishing this job, the xylene dissolving the last traces of the balsam. Here again care must be exercised not to use too much of this oil or the mount will be softened and perhaps the cover loosened. An especially good cleanser for the slide as a whole is a paste made by adding a few drops of xylene to powdered Bon Ami; turpentine has its advocates too. Sometimes slides become unsightly because of smears or stains on the glass, beyond the cover glass area, and these smears are often troublesome. Try first a washing in strong soap suds and warm water, not hot enough to melt the balsam. Rinse and polish with a soft cloth, taking care not to apply pressure over the cover glass region. Strong alcohol, acid alcohol, or a 5% solution of ammonia in water are all good and may work in particular cases, but none of these should come in contact with the thin rim of balsam where the cover glass edge and slide meet; they will turn the balsam milky. If these strong chemicals are used, rinse and wipe out of water.

**Labeling a Finished Slide.**—Aside from various methods of writing on glass already enumerated, any of which may serve as a permanent label if desired, there remains for discussion the permanent paper label, still far in the lead in popularity over other systems. Any sort of gummed label



may be used, but the serious microscopist should consider only the regulation *microscope slide labels*, expressly prepared for this purpose. They are slightly less than 1 by 1 in. square, have a narrow border of black or blue lines, and are

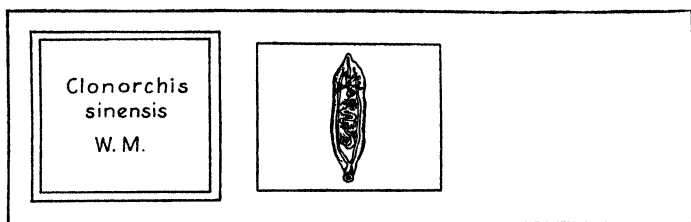


FIG. 45.—A finished and labeled slide.

furnished loose in a box, in a perforated roll like tape, or in perforated sheets in a book. These sheets are recommended if a typewriter is to be used to do the printing, since single labels are too small for easy insertion in a machine. However, most typewriter print is far too large for this work and, unless you happen to own a machine with miniature type faces, hand-printing is decidedly preferable. If the slide collection is to be at all extensive, it is good practice to have your personal labels printed by the supply house furnishing them. They use 6-point bold type to print your name across the top and residence along the bottom and leave ample room in between for all the rest, which you then letter by hand.

Use only a waterproof black India ink, and either a crow-quill, eagle quill, Spencerian No. 1 pen, or other make of the same fineness. Print neatly, using larger letters for the main heading, smaller ones for the less important data. If you cannot print well, this is a good opportunity to practice and learn, since printing is an accomplishment everyone should master. The few who own a small printing press and like type-setting as a hobby will naturally print all their labels.

Material to appear on the label varies widely according to the kind of slide and individual preferences of the

operator. Most important, of course, is to tell what the object on the slide is and, if not a whole object, to name the part represented. Thus the name of the organism and its part come first. There follows the type of preparation,

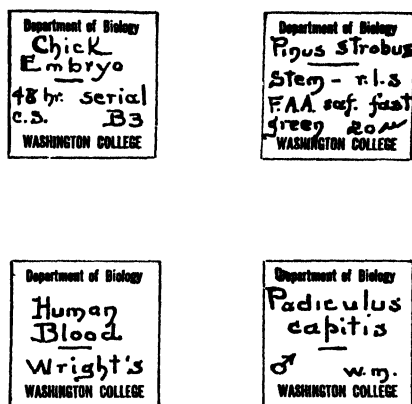


FIG. 46.—Examples of data appearing on slide labels.

whether whole mount, isolation, smear, or section. *Examples:* *Housefly* (large print, single line, at top), draw a line under this word, then add *wing*, and under this *w. m.* in smaller print, the “w. m.” standing for “whole mount”; *Esophagus*, *Homo* (the scientific name for man), *c. s.* (abbreviation for “cross section”).

When stains are used, they are frequently stated on the label and often the fixer as well. Those in commonest use have standard abbreviations well understood by the microscopist, thus, *Lung*, *Cat*, *c. s.*, *Zenker's* (the fixer), *H* and *E* (hematoxylin and eosin, the stains). If the mountant is balsam, this need not be stated; it is assumed. If some other mountant is employed, the name should appear on the label. Certain special processes or reagents may be included if desired, and many workers prefer to suffix the date and name or initials of the preparator if not already machine-printed on the label.

Some labels need few data, others a great many; sometimes two labels are needed, one at each side of the cover.

Figure 45 shows a cleaned, polished, labeled slide; Fig. 46 gives some examples of lettered labels.

Nothing is superior to saliva in moistening the gum of labels; we prefer to lick them in the time-honored way, rather than use any of various wetting appliances. Write the label first, let it dry well, then lick and press it down firmly all around, covering the tip of the finger with a handkerchief. Iron out completely or the label will not stick. Put the label to the left of the cover glass. This is a standard convention since, on microscopes equipped with a mechanical stage, the right margin must be clear in order to read a scale through the glass.

## CHAPTER 4

### PROCEDURES IN MICROTECHNIQUE

*In This Chapter:* classification of slide preparations; processes in technique—killing, fixing, washing, dehydrating, hardening, dealcoholizing, infiltrating, imbedding, sectioning, staining, destaining, clearing, mounting; dissecting instruments; optical aids for dissection; glassware and miscellaneous accessories.

**T**HUS far we have dealt either with temporary mounts or with permanent slides requiring no other preparation than clearing and mounting. Since the great majority of subjects need additional and often many separate steps for their completion, we shall, in this chapter, outline all the general technical operations as a basis for particular examples taken up in later chapters.

**Kinds of Slide Preparations.**—1. *Whole mount* or in toto mount: an entire object or a part of one, made without cutting into thin sections, as small worms, insects, embryos, or seeds; or a piece of paper, fabric, sand grains, finger-print, or chemical crystals; an insect spiracle, a mosquito stomach, a fish scale, a leaf, or a flower.

2. *Smears* comprise a special class of whole mount which, as the name indicates, are thin films of a fluid, with or without solid contents, such as soil or blood smears, and feces containing parasites or their eggs, such as would be made in a hospital laboratory in order to diagnose a disease of the digestive tract.

3. *Sections* are exceedingly thin slices of an entire organism or any of its parts, and are necessary whenever large, thick, or opaque structures are to be examined by transmitted light. Commonly prepared sections include such examples as hydra, earthworm; liver, heart, brain; plant stem, root, leaf, and ovary.

4. *Isolations* involve the tearing apart or separation of some elements from others in order to expose them to more detailed study and include outright *dissections*, as when the mouthparts of a grasshopper are carefully worked out of the head and mounted separately in a spaced arrangement on a slide (Fig. 79), as well as *teased* objects, for example, the lengthwise shredding of a muscle with needles in order to secure individual fibers for mounting.

No hard and fast lines can be drawn among these four classes of mounts. The mouthparts and muscle fiber slides are whole mounts as well as isolations and securing a mosquito stomach to mount entire requires a microdissection; yet the four terms are convenient in describing general types. Examples of all of these will be given in succeeding chapters.

### MICROTECHNIQUE

The many kinds of acts one must perform in order to turn out creditable slides of organic materials fall within four main divisions: fixing, sectioning, staining, and mounting. Each of these has a number of subdivisions, to be described in turn.

**Killing.**—If a living plant or animal is to be mounted whole, dissected, or sectioned, or if any of its parts are to be removed for such purposes, the first requirement is to kill the organism in a way that will not injure the structures wanted. Humane considerations must also govern the methods used with the higher animals. As employed in microtechnique, however, the term “killing,” a subdivision of the general process of fixing, applies to the individual cells of the organism, since each of these possesses life.

Small animals with thin permeable walls, as a daphnia, may be killed in such a way that all of the cells perish with the death of the organism as a whole; the same cannot be said of larger animals or plants.

Two general methods of killing are in use: slow and rapid. *Slow killing* is performed with narcotics and anesthetics and is used only on those animals whose expansion and relaxation after death are essential for natural mounts. As an example, one may add epsom salts slowly, a pinch at a time, to the water in which hydroids, as *Obelia*, are kept, the animals dying in a fully expanded state. Aquatic air-breathing snails may be asphyxiated by confining them in a covered jar completely filled with water, so that they have no access to air for respiration; they will expire with the body entirely expanded, relaxed, and protruding from the shell. Heat is a good killing agent in some cases, too, daphnias being dispatched by gradually heating the water in which they are living.

*Rapid killing* is better whenever possible and includes a great variety of methods, some of which are here listed. A hydra is best killed by flooding it quickly with a hot corrosive fluid, death overcoming the tiny animal before it has time to contract. Thin films of blood cells are killed and fixed by mere exposure to air. Ether or chloroform may be used with frogs, snakes, and many other animals, but as they usually die in contorted postures with strongly contracted muscles, other methods are generally preferred. Small lizards and snakes may be decapitated with scissors; larger snakes by smashing the head with a hammer or by drowning. Turtles are among the most difficult of all vertebrates to do away with, especially such large and tough customers as the snapper. Pouring ether down the throat and injecting it into the rectum seems to be about the best method so far discovered.

The larger butterflies and moths as well as small birds are easily killed by compressing the thorax between thumb and forefinger so that breathing movements are prevented. The slaughterhouse uses a sledge or knife with large mammals but in the laboratory, where such smaller species as the cat, rat, and guinea pig are most usually the type wanted, the quickest and most humane procedure is to gas

them in a small, airtight box, connected by rubber tubing to a gas jet. The insidious and deadly carbon monoxide gets in its work rapidly and painlessly.

Inasmuch as frogs are so widely used in histological studies, the preferred method of killing them, known as



FIG. 47.—Method of holding a frog for pithing. X indicates place to insert scalpel or needle.

*pithing*, will be detailed. Grasp a frog in the left hand, as shown in Fig. 47; hold it with its back up and head to the left of the operator, the forefinger holding and depressing the head, the thumb over the back, and the remaining fingers supporting the belly as well as preventing movements of the hind legs. By running the right forefinger back along the middle line of the head from between the eyes to a point about opposite the two ear drums, the fingernail will easily locate a depression that marks the end of the skull and the place where it articulates with the first vertebra. This is the site for pithing. With a small sharp scalpel or a heavy needle in a holder, penetrate this spot, plunging the instrument in quickly and moving it slightly back and forth sidewise, in this way severing the spinal cord from the brain. The whole operation can be performed almost instantaneously and is equivalent to decapitating or guillotining as far as any sensation on the part of the frog is

concerned. If the brain is wanted for sectioning, sever the head from the body and remove the brain; if not wanted, complete the pithing by next destroying the brain by running a needle or wire through the incision first made and working it back and forth so as to mash up and obliterate the brain. The instant the spinal cord is severed, no sensations of pain can be transmitted to consciousness from any part of the body in rear of the cut; any subsequent movements of the legs are reflex and do not indicate any suffering on the part of the hapless victim.

With all plants and with larger animals, killing of the tissues themselves is performed by the fixer, the reagent used in the next step, so that killing and fixing become one and the same operation, though with different results. Hence, immediately upon taking the life of a larger animal, the specimen should be opened, the required organs or tissues removed, washed for an instant in normal saline solution to get rid of blood, hair, or other foreign material, trimmed to proper size if necessary, and then at once placed in the fixer, which kills the individual cells.

**Fixing.**—This is one of the most important of all steps in technique and must be done thoroughly and correctly if good results are to be achieved. Fixing combines a number of separate acts in one. As we have seen, the fixing solution *kills* the individual cells; secondly, it *preserves* (fixes) them against future solution or decay; thirdly, it imparts changes that bring about *optical differentiation* by altering the refractive indices of various parts so that these parts refract transmitted light to different degrees, and thus present differences to the eye in using the microscope—differences that are not apparent in fresh tissue. Fourth, most fixers initiate a later step in technique, that of *hardening* the object.

A good fixer should do all four of these things. As might be suspected, few if any chemicals have all of these capacities developed to the proper degree; hence, most fixers are



mixtures of different chemicals, some to do one piece of work, others for different jobs. Such mixtures are usually named after the inventor of the particular formula, *e.g.*, Bouin's fluid, Flemming mixtures.

To kill tissues quickly and thus prevent post-mortem changes, a fixer must be able to *penetrate* rapidly. Acetic acid is widely used in mixtures to do the penetrating, carrying other ingredients in with it, but has the disadvantage of causing marked swelling. This may be offset by combining with the acid some other reagent that will shrink and hence neutralize the swelling effect without interfering with penetration. Corrosive sublimate will do this and at the same time preserve and give good optical differentiation.

The preservers are mainly salts of metals and act by forming insoluble precipitates in combination with the tissues, so that they cannot be washed out or dissolved later. Potassium bichromate preserves, differentiates, and hardens admirably but acts very slowly, so needs partnership with a penetrator. Of all the simple fixers (not mixtures), only alcohol and formalin have met with wide usage. Very strong alcohol, as 100%, is preferred for this work; with formalin 10% is the usual strength employed and makes a very satisfactory fixer for certain objects.

**Washing.**—A thorough washing out must follow both fixing and staining and is done to check the action and remove excess chemicals. After fixing, it is quite essential to flush well, usually with water, and a good general rule—though not a universal one—is to wash for the same length of time as for fixing.

**Dehydration.**—This is the removal of water, following washing. Most fixers are aqueous (watery) solutions and, since water would gradually deteriorate even well-fixed tissues and is moreover not miscible with paraffin for imbedding or balsam for mounting, it must be eliminated. As

explained by a previous experiment on miscibility and the formation of convection currents, dehydration is accomplished gradually, as a rule; the time-honored method is to pass the material through a series of alcohols of increasing strengths, each step replacing more and more of the water until finally, in absolute alcohol, none is left. This step is performed after fixation and again after staining.

**Hardening.**—If objects are to be cut with a knife in the preparation of thin sections, it is advantageous to harden them considerably, fresh tissue being generally too soft to submit to cutting without a destructive mashing or other distortion. As stated, most fixers also perform hardening; the alcohol used in dehydrating is likewise excellent for this purpose. Hence hardening, although actually a separate step, is not performed separately but is automatically accomplished during the routine of fixing and dehydrating.

**Dealcoholization.**—Here is another process that is taken care of incidentally, though it is really a separate item. Alcohol will not mix with either paraffin for sectioning or with balsam for mounting and must be got rid of. Clearers such as xylene dealcoholize while they clear; hence both steps are performed as one.

**Infiltration.**—Standard methods of sectioning involve reinforcing the object with some medium which is fluid at one stage but which turns more or less solid later, as on cooling or on exposure to air. If such a medium can be made to penetrate all parts of the organ thoroughly, adequate support will be given and sections can be cut without crushing or tearing. Melted paraffin, for example, kept fluid in a low-temperature oven, can be made to infiltrate all parts of the object; later it turns to a solid when removed from the oven. During infiltration proper, the clearer in the tissues is replaced gradually by the imbedding medium.

**Imbedding.**—After a complete infiltration, the object is next imbedded in a fresh supply of the medium which is then solidified. When cold and hard, a block of paraffin containing the tissue is ready to cut.



FIG. 48.—Photomicrograph of section of cat ovary, unstained. Very little of the structure may be seen. (24 X.)

**Sectioning.**—In producing thin sections the cutting process itself is an important and delicate operation and involves a certain amount of skill gained through practice. The implements vary from a safety-razor blade to complex sectioning machines termed “microtomes.”

**Staining.**—Nearly all the smaller organisms, as well as all thin sections, appear more or less colorless when viewed through the microscope with transmitted light. This means that the various parts one wishes to distinguish will be invisible, indistinct, or at best made out from one another with great difficulty and uncertainty (Fig. 48). With the aid of artificial color, objects are made to stand out prominently. Because of the optical differentiation of the several parts which was introduced by the fixer, some structures will take the dye, others will not, or some will take it to a different degree than will others (Fig. 49). Staining, then,

is done not for purposes of beautification but to bring out details of objects and to distinguish between separate parts.

The chemicals used may be either acids or bases and act by forming insoluble precipitates or by combining chemi-

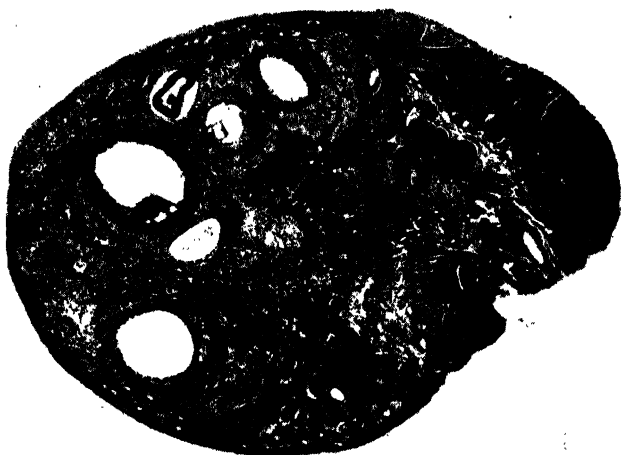


FIG. 49.—Same material as in Fig. 48, but the section stained with hematoxylin and eosin. Shows how staining provides contrast and colors all parts distinctly.

cally with the tissues to make what the textile worker calls a “fast” (*i.e.*, fastened onto) combination. Most biological dyes, known as “stains” rather than dyes, will resist washing out by water or alcohol, though they may be extracted with an acid.

Whole mounts are generally *single stained*, with one color only, a procedure often referred to as *in toto staining*. Sections may be single stained also, but it is much better and the almost universal practice today to use two dyes, the process called *double staining*; the first application is the *stain*, the second one the *counterstain*. If one of the two reagents is an acid and the other a base, a selective action can be obtained: one dye will affect only certain structures; the second will color other parts. For example, a favorite

routine combination with animal sections is hematoxylin (pronounced hē-muh-tōk'-sī-līn) and eosin. The hematoxylin, a basic dye, colors nuclei of cells purplish blue; the eosin, an acid stain, imparts a rosy-red hue to cytoplasm. An epithelium, containing many large nuclei, will appear blue; right alongside of this layer, the connective tissue with its preponderance of cytoplasm will be colored red. Such contrasts greatly facilitate interpretation and identification.

**Destaining.**—A few objects, such as frog eggs, are so darkly pigmented as to need extraction rather than addition of color. In such a case this decolorizing process is often termed *bleaching*. Principally, however, destaining is employed when tissues are overstained and need some of the excess removed to tone down the color from a violent, disagreeable hue to a more pleasing and translucent tint. One of the staining methods is termed *progressive staining*, in which the tissues are stained until of the right intensity, then the action is stopped and no destain used. A more exact and accordingly more widely practiced method is that of *regressive staining*, in which the material is purposely overstained until all parts are deeply affected, then a destain is employed to extract color in certain parts or from the whole object until it is of the correct depth.

**Clearing.**—As we have seen in the previous chapter, the use of an essential oil renders objects translucent and hence in suitable condition for study by transmitted light, xylene being the favorite clearer. It has also been explained that this act accomplishes dealcoholization and prepares the tissue to receive either paraffin for infiltration or balsam for final mounting, clearers being miscible with both.

**Mounting.**—This is the imbedding of the object in balsam or other mountant, on a slide, and the adding of a cover glass. We have already performed simple mounting and are shortly to practice mounting within cells. After being dried

and hardened, the slide is cleaned, labeled, cataloged, and stored, all of which are minor steps but quite essential to careful work.

**Summary.**—Now, reviewing the foregoing technique operations, the sequences of steps are based on a fundamental reasoning which, once mastered, should become second nature to the operator and there should be no occasion for mistakes. If you plunge a slide from xylene into water, you can expect only one result, knowing the principle of miscibility.

Remember that organic materials have to be fixed and that nearly all fixers are solutions of solids in water. Because of this, the washing out must be done with water or a weak alcohol. Actions such as fixing and staining must be checked when completed; hence a washing out of excess reagents is always indicated.

Starting in water, tissues or slides must be run up through a graded series of alcohols to dehydrate them without injurious sudden changes. After sectioning in paraffin, knowing that paraffin is not miscible with either alcohol or water and that staining in a watery solution is coming up, the obvious procedure is to dissolve the paraffin with xylene, then pass down through the alcohol series in reverse order, to water; apply stain; wash out excess stain and ascend the alcohol ladder again to xylene; then mount in balsam. A moment's thought in advance of any operation should tell one the proper step to take; it soon becomes automatic to do the right thing at the right time.

Upon deciding to prepare material by using any technique with which you are not already thoroughly familiar, it is advisable to lay out a definite program at the start. As seen in the schedules that follow, titles to steps may be written down the left margin of a sheet of paper; then the selected reagents and time durations filled in. These titles may also be mimeographed or machine-printed on sheets or cards, thus providing an adequate supply as well as permitting filing for future reference.

## EXAMPLES OF TYPICAL SCHEDULES

Object: *Planaria*, a small flatworm.

Kill: 1 % chromic acid, 5 min.

Fix: formol-acetic-alcohol, 24 hr.

Wash: 70A (water unnecessary, fixer contains alcohol).

Store: fresh 70A until ready for next step. Time of washing and storing immaterial.

*Whole Mount*

Hydrate: 50A, 10 min.

water, 5 min.

Bleach: hydrogen peroxide, until pale.

Wash: water, 1 hr.

Stain: alum cochineal, 24 hr.

Wash: water, 15 min.

Dehydrate: 35A, 10 min.

50A, 10 min.

70A, 10 min.

Destain: acid 70A until staining is correct.

Wash: fresh 70A, 2 changes, 10 min. each.

Dehydrate: 82A, 15 min.

95A, 15 min.

100A, 15 min.

Clear: xylene, 15 min.

Mount: balsam.

*Sections*

Dehydrate: 82A, 15 min.

95A, 15 min.

100A, 15 min.

Clear: xylene, 30 min.

Infiltrate:  $\frac{1}{2}$  xylene,  $\frac{1}{2}$  melted paraffin, 30 min.  
melted paraffin,  
3 changes, in oven, 45 min. each.

Imbed: fresh melted paraffin.

Section: paraffin method.

Affix: fasten sections to slides.

Deparaffin: xylene, 15 min.

Hydrate: 100A, 1 min.

95A, 1 min.

82A, 1 min.

70A, 1 min.

50A, 1 min.

water, 2 min.

Stain: hematoxylin, 30 min.

Wash: water, 5 min.

Destain: acid 35A, until red.

Rinse: alkaline 35A, until blue.

Dehydrate: 50A, 1 min.

70A, 1 min.

82A, 2 min.

95A, 3 min.

Counterstain: eosin,  $\frac{1}{2}$  min.

Rinse: 95A, 1 min.

Dehydrate: 100A, 5 min.

Clear: xylene, 10 min.

Mount: balsam.

## CONCERNING DISSECTING INSTRUMENTS

The ordinary grade of school or college biology dissecting kit, costing in the neighborhood of \$2, will serve most needs in making slides, or the operator may buy instruments a few at a time, as needed. We suggest the following items.

**Knives.**—A knife for anatomical dissection is termed a “scalpel”; there are many sizes and styles of these, some with ebony (wooden) handles (Fig. 50), others of all-metal construction. One small scalpel for fine work and another coarser and larger would be sufficient. Recently there has come on the market the Bard-Parker knife, consisting of a



FIG. 50.—Scalpel.



FIG. 51.—Bard-Parker knife, with removable blades.

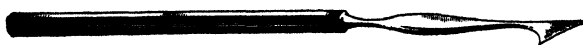


FIG. 52.—Dissecting needle.

permanent steel handle and packages of blades of various sizes and shapes. These lock onto the handle, are quickly removed and discarded when dulled like safety-razor blades, and the same handle will serve for both fine and heavy blades (Fig. 51). The operator may manufacture his own knives by clamping a new safety-razor blade in a vise, breaking it lengthwise to secure a half-blade, grinding down the broken edge, and mounting it by riveting, wrapping, or cementing in a length of wooden stick of convenient dimensions. Minute knives may be made by hammering flat the end of a small piece of steel wire, grinding and honing a



sharp edge, tempering, then mounting it in a handle such as a matchstick or wooden dissecting needle handle. Very small commercial models (Fig. 52) are frequently termed "dissecting needles."

**Razors.**—For sectioning, one needs a good razor and can use either the old-fashioned kind (Fig. 53), a professional microtome knife, or safety-razor blades. Some firms, as Gillette, manufacture an especially fine grade of wafer-thin blade designed to be mounted in a special blade holder for insertion in a machine microtome. The Gits Molding Corporation, Chicago, manufacture a folding pocket carrier for a safety-razor blade, excellent for freehand and well microtome sectioning, termed a "Razor-Nife" (Fig. 54)

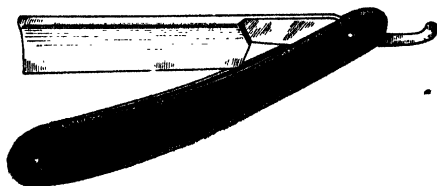


FIG. 53.—Sectioning razor.

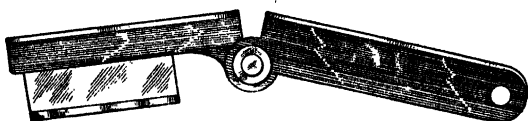


FIG. 54.—Gits Razor-Nife.

and available in both long and short lengths. Any device using safety-razor blades has the advantage of doing away with the exacting and tedious performance of sharpening and honing, necessary every so often with permanent blades.

**Scissors.**—This is the most expensive of dissecting items since cheap scissors are utterly useless for any fine operations. If much work is to be done, two pairs will be needed, coarse and fine. A good quality of manicure scissors will serve nicely for the fine pair, with either straight or curved

blades. Two types of regulation dissecting scissors are shown in Fig. 55.

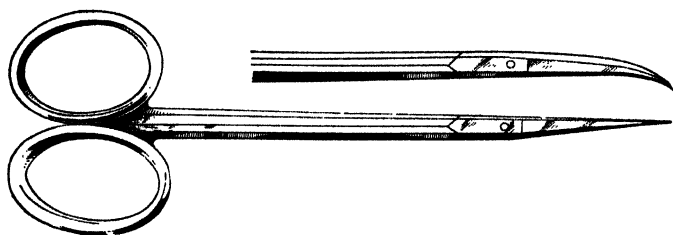


FIG. 55.—Two styles of dissecting scissors.

**Forceps.**—Here again, one should have two pairs—coarse and fine—the latter with either straight or curved blades

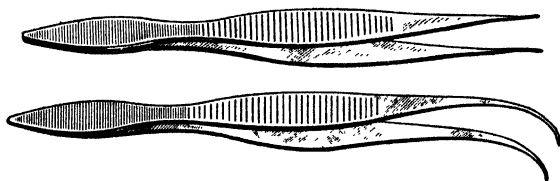


FIG. 56.—Two forms of forceps.

(Fig. 56). Cheap forceps, called “tweezers” by some, in which the nibs will not meet perfectly, are a waste of money.

**Cover glass forceps** are not absolutely necessary but they will be found most useful. Unlike other forceps, these are reversed; they remain closed when released and must be pinched to open. Of the several commercial types, Fig. 57 shows two. When laid on a table, they will hold a cover glass perfectly flat and elevated above the table where it is kept clean and convenient to work upon in making smears; or they will hold a glass ready for sealing mount, for flaming, and for other purposes.

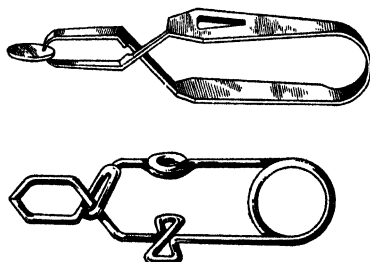


FIG. 57.—Two types of cover glass forceps.

**Seeker.**—This is an elongate metal instrument (Fig. 58) with the handle at the center. One end tapers to a fine

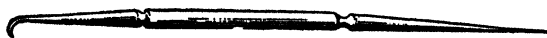


FIG. 58.—Seeker.

sharp point, like a coarse needle, the other to a crescent-shaped hook with a sharp inner cutting edge, like a miniature sickle. It is a highly useful instrument in all fine dissections, for moving parts about while seeking a certain object, for cutting, tearing, teasing, and other operations. One is sufficient.

**Needles.**—The regulation coarse steel needle mounted in a wooden handle (Fig. 59), as supplied with most biology



FIG. 59.—Wooden-handled dissecting needle.

kits, is satisfactory. By splitting or drilling any round stick of convenient size, similar needles may be made, using cement, sealing wax, tape, shellac, etc., to hold securely

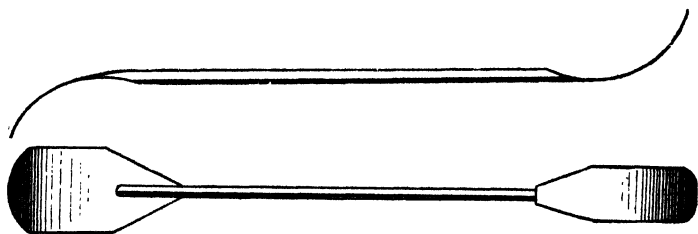


FIG. 60.—Double-ended section lifter, side and surface views.

any size of steel needle one chooses. Also there are bone or metal adjustable needle holders (handles) for the insertion of sewing needles of various sizes, where they are held by a chuck. Two needles, one for each hand, are desirable for fine work, as on insects.

**Section lifters**, available in many styles and sizes (Fig. 60) are used in lifting up sections and are also very handy

in whole mount work to transfer objects from one reagent to another or to a slide. They can be made as shown in Fig. 61 by cutting them out of sheet brass or aluminum and bending up the tip to a 45-deg. angle.

#### OPTICAL AIDS FOR DISSECTION

Fine dissections are facilitated by magnification for the vision, preferably by means of some device that will leave both hands free. There are a great many such appliances, ranging from hand lenses and magnifiers supported in a stand or tripod, through loupes to dissecting microscopes and finally binocular microscopes, as described in Chapter I.

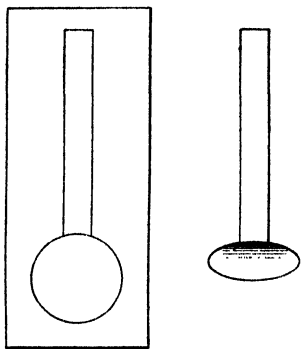


FIG. 61.—Method of making a section lifter.

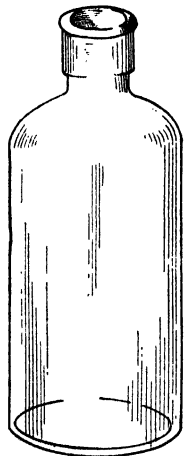


FIG. 62.—Narrow-mouthed reagent bottle for cork stopper.

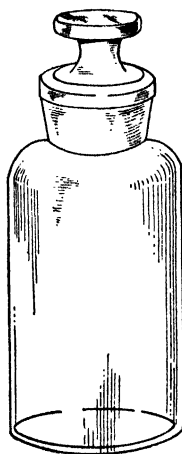


FIG. 63.—Wide-mouthed reagent bottle, ground-glass stopper.

#### MICROTECHNICAL GLASSWARE

Laboratory requirements over a long period of years have established certain forms of glassware as most suited to

particular uses; whether you follow this custom or employ substitutes from the chain store or the pantry shelf, you should at least know the names and appearance of these standard items.

**Bottles** are manufactured in an endless array of shapes, sizes, and colors. We have already noted the use of wide-mouthed collecting bottles (Fig. 21) and such specialized forms as the balsam bottle (Fig. 37) and alcohol lamp (Fig. 35). Remaining to be mentioned are the reagent bottle, obtainable in many sizes and in both narrow-mouthed (Fig. 62) and wide-mouthed (Fig. 63) styles; the first for fluid reagents such as fixing solutions, alcohols, and staining solutions; the second for dry chemicals, for example, bichromate of potassium crystals or sodium chloride. Also these bottles may be provided with plain mouths for cork stoppers (Fig. 62) or with the more expensive ground mouths for



FIG. 64.—  
Shell vial,  
screw cap.

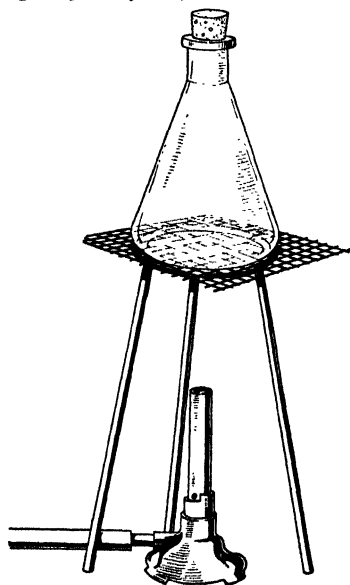


FIG. 65.—Bunsen burner, tri-  
pod, wire gauze, and erlenmeyer  
flask.

ground-glass stoppers (Fig. 63), the latter type being essential for either solid or liquid contents that readily absorb water vapor from the atmosphere or evaporate with greater speed than ordinary. The microscopist needs to add many bottles to his equipment from time to time

in order to house his growing collection of both new and used reagents.

**Vials** are small, straight-sided bottles fitted with either corks or screw caps (Fig. 64); they are very useful for storing small specimens, such as preserved or fixed insects, worms, protozoan cultures, fern prothallia, and the like; also for dry stains and small quantities of various chemicals.

**Flasks** are containers in which reagents are to be heated, shaken, mixed, or otherwise treated; at least one will be needed in making up fixing solutions. The chemistry laboratory stocks many styles and sizes, but the type here illustrated (Fig. 65), known as an "erlenmeyer flask," is most suited for our work. Those made of pyrex glass are best, and we suggest either the 500- or 1,000-cc. size.

**Beakers** are smaller but have similar uses, such as mixing reagents, stirring, or merely for pouring since they have a convenient spout (Fig. 66).

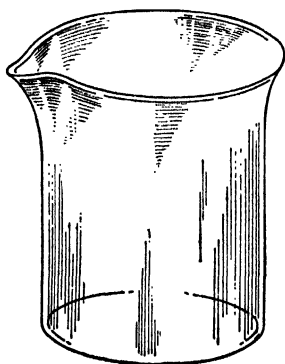


FIG. 66.—Beaker.

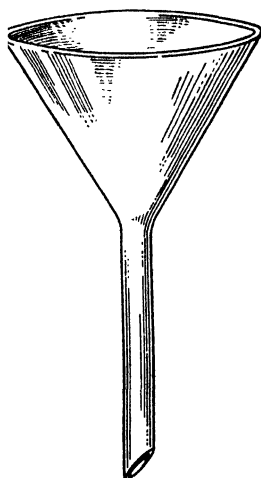


FIG. 67.—Funnel.

**Funnels** are necessary for filtering operations and handy in filling large bottles, as in the making up of stock solutions

and grades of alcohols. One, of average size, should be sufficient (Fig. 67).

**Graduated cylinders** are required for measuring fluids in making up formulas and have already been discussed (Fig. 32).



FIG. 68.—  
Test tube.

**Test tubes** (Fig. 68) are elongate, round-bottomed vials in which materials are mixed, heated, stored, or otherwise processed. We shall meet them intimately in the chapter on bacteria. The average operator should put in a dozen at a time as they are not expensive. They come in plain or pyrex glass in several sizes, with or without corks.

**Trays** may be of glass (Fig. 69), metal, or enamel. They are useful for staining by the drop method, examining materials, dissecting, and are essential in photographic developing. Dissecting trays are special forms of japanned metal with wax bottoms, into which pins may be pushed to hold parts of the specimen.



FIG. 69.—Glass tray.

**Battery jars** are the best form for maintaining cultures, as already discussed (Fig. 23). They are inexpensive and obtainable in several sizes.

**Crystallizing dishes** are low flat cylinders (Fig. 42) useful in the field, in chemical crystal work, and for smaller cultures. They are available in many sizes, plain or pyrex glass.

**Specimen dishes**, also called "biological finger bowls" (Fig. 70), are very handy for small cultures, especially in subculturing for pure cultures, as in maintaining a definite species of protozoan over a period of time. They stack one upon another and so need no lids.

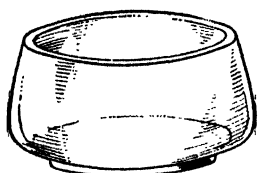


FIG. 70.—Biological finger bowl or specimen dish.



FIG. 71.—Petri dish.

**Petri dishes**, still smaller forms for culturing (Fig. 71), are universally used in bacteriology, including work on yeasts and molds. Such a dish is essentially a circular glass box of shallow depth, one-half forming a lid for the other and fitting loosely. They are handy with protozoa and in a number of staining operations.

**Syracuse watch glasses** (Fig. 72) are flat receptacles for work with all small organisms. Living hydras, worms, rotifers, microcrustacea, and the like may be placed in one of these dishes on the stage of the microscope for observation; they are also useful in fixing and staining. They stack, like the specimen dishes already referred to.

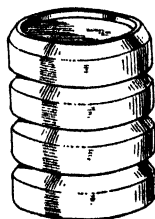


FIG. 72.—  
Syracuse  
watch glasses,  
stacked.

**Stender dishes** (Figs. 19, 20) have already been discussed. They are unexcelled for storage of slides and covers, as general containers of such articles as cotton, wooden splints, used paraffin, for many fixing and staining operations, and for a wide variety of other uses.



**Dropping bottles** (Fig. 73) have been deferred to this point in relation to the next item. Equipped with a pipette fitting by a ground-glass connection, they are very fine for keeping small quantities of reagents that are to be used one or a few drops at a time, as iodine solution and many stains.



FIG. 73.—  
Dropping  
bottle.

**Pipettes** (Figs. 24, 25) are required when doing culture work or in all cases where small quantities of a fluid must be delivered elsewhere.

**Coplin staining jars** (Fig. 74) are only one of the many types of glassware on the market designed especially for staining slides, with particular reference to handling more than one slide at a time. The glass lid is ground on to prevent evaporation while in use, and the sides are slotted so as to take microscope slides containing sections. By placing the slides in pairs, back to back, as many as ten can be accommodated at once. Staining dishes of still larger capacity are available, but the independent worker who is not interested in quantity production will find the coplin jar adequate.

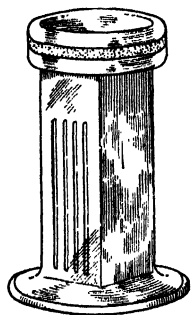


FIG. 74.—Coplin  
staining jar.

**Substitutes.**—Most housewives save their cheese glasses and use them for serving the morning glass of fruit juice. The microscopist can well do likewise since these glasses make very good tall stenders. Coasters that are almost duplicates of syracuse watch glasses, low cold-cream jars which resemble flat stenders in size and shape, jelly glasses, jars for fruits and preserves, and many other items can be picked up at ten-cent stores. Save all your bottles from proprietary drugs and the like to use as containers for reagents.

## MISCELLANEOUS ACCESSORIES

We shall have occasion to mention pieces of apparatus and various items of equipment such as a turntable, oven, sterilizer, and the like in succeeding chapters, as our needs arise. At this point there remain a number of odds and ends to suggest.

**Boxes.**—Save your safety-match boxes; they make excellent receptacles for small dry specimens. Cardboard pillboxes are even better, since the lids fit tightly. Ointment boxes of tin or aluminum (Fig. 75) are widely used to house specimens and small accessories. Keep all the small boxes of specimens of the same nature, *e.g.*, sands, in a single larger receptacle, such as a cigar box.

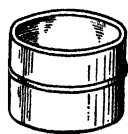


FIG. 75.—  
Metal ointment box.

**Envelopes** are preferred for these purposes by many. The manila coin envelope is good; likewise the newer, transparent glazed paper or cellophane type.

**Capsules** are unexcelled for the storage of fine and valuable material, such as sands and earths containing diatoms, foraminifera, and radiolaria, or hairs of unusual mammals. Veterinary capsules are of suitable size for fairly large objects.

**Labels,** adhesive, blank. Assorted sizes are needed for many purposes.

**Brushes** of several styles and sizes should be at hand for cleaning glassware. These are generally known by the particular glassware item for which they were designed, as test-tube brushes, bottle brushes, and so on. In addition, several small, fine brushes will be needed for transferring delicate specimens from a reagent to a slide, in mounting

diatoms, handling embryos, and the like. The camel's-hair brush (Fig. 76) is best for its softness; if a somewhat stiffer tuft is desired, secure one or more of the sable brushes used by artists. Smoker's pipe cleaners are splendid for running through pipette barrels.

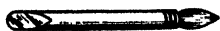


FIG. 76.—Camel's hair brush.

**Inoculating loops and needles** are essential when working with bacteria. Those made of nichrome wire are fully as serviceable and much less expensive than platinum types.

**Glass and rubber tubing** are used in short lengths in making many forms of laboratory apparatus.

**Corks and rubber stoppers** in several sizes should be kept on hand.

**Filter Paper.**—At least one package of diameter appropriate for the size of funnel selected is needed.

**Burners.**—If gas is available and to be used in heating reagents, a bunsen burner (Fig. 65) is an important item of your equipment. Many styles of electric heaters and hot plates are likewise available.

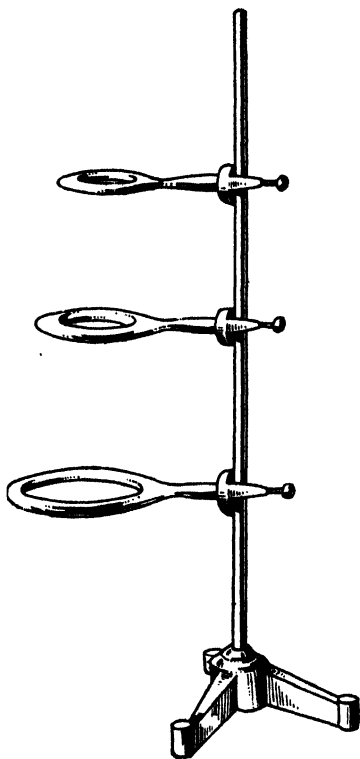


FIG. 77.—Ring stand.

**Tripods** elevate a flask or beaker above the bunsen burner for cooking operations. A piece of wire gauze should be

placed across the top of the tripod as a rest for the glass container (Fig. 65).

**Ring stands** support funnels and other pieces of equipment (Fig. 77).

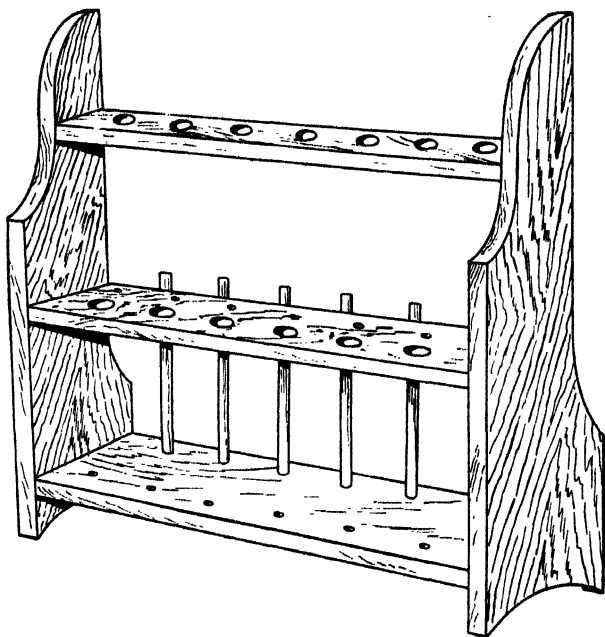


FIG. 78.—Test-tube rack.

**Test-tube racks** are supports for these tubes during use as well as for drying after cleaning (Fig. 78).

**Thermometer.**—The minimum requirement is one centigrade laboratory model for use with ovens, sterilizers, and incubators.

## CHAPTER 5

### PROCESSED BALSAM MOUNTS

*In This Chapter:* whole mounts of insects and their parts; the potashing technique; radula of a snail; leaves and pollen; starch grains.

**W**HOLE mounts are processed in a variety of ways depending on the subject and the purpose of the preparation. Some materials are mounted dry, in air; others in glycerin; the majority in balsam. Certain of them require the construction of a ring or cell mount; many insect objects must be potashed for best results. In many cases no staining is necessary; in others a stain is preferable. Most whole mounts are to be viewed by transmitted light and hence are made as transparent slides; in a few cases processed opaque mounts to be used with reflected light are better. Examples of each of these styles will be given.

**An Insect Whole Mount.**—Search a garden plant, such as a rosebush or a sweet-pea vine, for specimens of those tiny but exceedingly abundant and damaging insects known as “aphids” or plant lice. They are very delicate, wingless creatures and must be handled with care. Shake some of them into an alcohol bottle; when ready to mount, remove them from the alcohol with a brush, avoiding handling with the fingers or coarse instruments. Shake others into a cyanide jar and keep them dry until ready for mounting.

*Method 1:* Place a dry aphid in cedar oil for 1 hr. Remove it with a brush or section lifter and place the specimen on its back on a scrap of filter paper. Add one drop of medium-thick balsam to the center of a clean slide and invert this slide over the aphid, pressing down gently. Invert again,

spread the insect's legs to a symmetrical position, add cover glass props, then a cover. Turpentine may be used in place of cedar oil. (See also Chapter 15 for other reagents.)

*Method 2:* Dehydrate an alcoholic specimen, as explained below, then pass through xylene or cedar oil for 10 min., and mount in balsam, positioning the legs with needles and using cover props. When needles or other instruments are to be used in balsam, moisten them first with the clearer to prevent their gumming in the mountant.

**Completing Dehydration.** *Method 1—In the Alcohols:* With a brush, transfer the aphid from the 70A of the killing or collecting bottle after 1 hr., to 82A and then 95A, 1 hr. each. To get the specimen from 95A, the pure commercial form, into xylene, one method is to complete the dehydration in the alcohol series by going through absolute alcohol, 100A, 15 min., then xylene or cedar oil, as above.

Absolute alcohol is an expensive reagent to purchase but may be made in the home laboratory by several methods. A regular chemical laboratory would first distill the 95A, but that is not necessary. Select a glass-stoppered bottle of about pint size and grease the stopper lightly with vaseline so as to exclude all air when the bottle is closed; this alcohol has so great an affinity for water that it will pick up moisture from the atmosphere unless this precaution is followed. Always keep the bottle tightly stoppered except when actually pouring 100A from it. Fill the bottle nearly full with 95A, then add a few lumps of calcium oxide (unslaked lime). This chemical will take the remaining water from the alcohol, slaking the lime. Enough should be added to leave an unslaked margin of safety.

Copper sulphate is another salt used for this purpose, the anhydrous white powder, which is added to the 95A. This turns the copper sulphate blue and more is added until this change ceases to occur. If only the blue crystals can be obtained, heat them to drive off their contained water until a white powder results.

Some workers prefer to prepare their 100A in one bottle, then filter it off into the final container, doing away with the dehydrating chemical. Others place the dehydrator in the final bottle then, when wanted for use, the 100A is carefully decanted (poured off) without disturbing the salts in the bottom of the bottle. From time to time, test the purity of the 100A by mixing a few drops with a like quantity of turpentine in a watch glass. If there is any water in the alcohol, the mixture will turn milky, and more dehydrator must be added to the stock bottle.

*Method 2—In the Clearer:* Instead of completing dehydration in the alcohol series, it may be done in the clearer; for this purpose nothing is better than carbolxylene. The carbolic acid has a great affinity for water and will remove any that remains in the object; in the words of the microscopist, carbolxylene will clear from 95A. It is good practice, especially with delicate objects, to pass from this mixture into pure xylene before mounting. In the case of the aphid specimen, transfer it from 95A to carbolxylene and then into xylene, 10 min. each. Anilin oil is another reagent that will clear from the higher alcohols (70 to 80%) and should be followed by bergamot oil before mounting.

*Method 3—In the Mountant:* Finally there are a few mounting media that are miscible with small amounts of water, thus rendering a complete dehydration unnecessary. They dehydrate, clear, and mount, all in one. Such are euparal and euparal vert (Flatters & Garnett; American agents, The Arthur H. Thomas Co., Philadelphia) and diaphane (Will Corporation, Rochester, N. Y.). From 95A, transfer the aphid to a slide and cover with a drop of euparal or diaphane. Arrange the legs, put on cover glass props, and then the cover glass.

Either method 1 or 2 is recommended for whole mount work with objects of any considerable bulk, such as insects or worms; method 3 is applicable more especially to blood smears and thin sections but is listed here in order to complete the topic of final dehydration.

A large number of insects and their parts are mounted by the foregoing schedule, with dehydration completed by either method 1 or 2; small flies and bees, flea beetles, lice, and such insect relatives as mites, ticks, and small spiders; antennae, legs, eyes, spiracles, ovipositors, and the like.

**Potashing an Insect.**—Many insects and their parts are so darkly pigmented that they do not make very satisfactory whole mounts by the simple method given thus far; the final result is too dense and opaque. Try the leg of a housefly and note that the mount is so very black that not much detail can be observed by transmitted light. The potash technique overcomes this objection and gives such superior results that most workers prefer to potash nearly all insect preparations save such very small and delicate specimens as aphids, thrips, and a few others. Most antennae and legs are greatly improved by this method.

*Potashing* involves a caustic alkali that will destroy all of the internal contents and leave only the outside of the insect or part intact, removing the muscles as well as the digestive, reproductive, and other internal organs. It must be remembered that an insect has his skeleton on the outside of his body, not on the inside as with human beings, and this framework, called technically the exoskeleton, is composed of chitin (kī'-tŷn) a substance allied chemically to horn, nails, and hair. It is resistant to the corrosive action of potash and remains unblemished through this severe treatment. The exoskeleton is all that is wanted in most whole mounts since the internal anatomy is best made out by dissections and cross sections. A well-prepared insect whole mount then, in the majority of cases, is no more than the flattened exoskeleton, emptied of all internal parts, and is hence much more transparent than an unpotashed preparation.

Caustic soda is sometimes used, but technicians on the whole prefer caustic potash (potassium hydroxide) in a 10% solution in tap water. You can purchase a stick of this



chemical and prepare your own solution, or get it ready made from the druggist. It is highly corrosive and must be handled with care.

Two methods of potashing are in vogue: (1) a prolonged soaking in the solution at room temperature and (2) a much shorter treatment in the same reagent by boiling. The latter is quicker but must be watched carefully and needs a bit of practice in order not to go too far and so destroy everything.

For this experiment, capture several large ants, small roaches, hog lice, mosquitoes, or other insects of medium size that are fairly heavily chitinized, that is, having a rather dense exoskeleton, more or less impervious to light. Place black ants, mosquitoes, and any other very darkly colored specimens into a bleaching solution, which may be a 3% peroxide of hydrogen, 12 to 24 hr., diaphanol (from E. Leitz, Inc.); or employ the following method with chlorine, a powerful bleaching agent. Into a test tube place 2 g. of potassium chlorate, then add 10 drops of hydrochloric acid. As soon as the green fumes of chlorine gas are seen, add 30 cc. tap water, then put in the specimens, which will bleach in one or a few days. Avoid inhaling any of these dangerous fumes. The commercial preparation chlorox is also a good bleacher.

Rinse these insects in water, then (along with those that required no bleaching) place them in cold 10% potash solution and leave for 24 hr. The lid of a baking-powder can makes a good receptacle for this work, but any glass vessel may be used. The tin lid is a good implement when potashed specimens are to be boiled; try a few of your insects this way too, heating them over an alcohol lamp for 30 min. In all cases, at the end of the required time, remove the specimens and wash them well in several changes of tap water to get rid of excess potash. Perhaps it is safest, as recommended by many technicians, to go next into concentrated acetic acid for an hour, to neutralize any remain-

ing potash as well as to soften and swell the insect, though this step can often be omitted.

Now use the handle of a wooden dissecting needle, a round matchstick, the handle of the camel's-hair brush, or any such small cylinder and begin gently rolling the specimen from head to tail, like rolling out dough with a rolling pin. The purpose is to expel the internal contents through the anus; if properly done on material that has been sufficiently potashed, the rolling should work perfectly, clean out the insect completely, and leave nothing but the exoskeleton, without any injury such as detached legs, broken wings, or ruptured abdomen. If unsuccessful, try again, varying the length of potashing or using greater care in rolling. If the specimen resists such treatment and the interior parts are not expelled, it has been insufficiently potashed; if it goes to pieces, it has been overpotashed. Only experience will teach one the correct length of time for the process, which depends on the sizes and permeabilities of various objects.

Now the preparation must be positioned and hardened. It is first rinsed well in water; from now on, greater than ordinary caution must be exercised in handling the insect since it is in a more fragile condition and is easily ruined by harsh or clumsy manipulation. Pour off the water in the container and slowly add fresh water; agitate this water gently by sucking it in and out of a pipette; repeat until the water is clean and free from debris. Get beneath the object with a section lifter or blank slide and lift it up, depositing it in a flat stender or syracuse watch glass containing 50A; then *position* the specimen, by which is meant straightening the body, adjusting the wings, and separating the legs into a lifelike and symmetrical posture. If there is a proboscis or other long mouthparts, as with a mosquito (which, by the way, should be mounted lying on its side), it should protrude downward, free from any overlapping by the forelegs. Take a fine needle and with the aid of a hand lens, slit the sheath of these mouthparts and separate the lancets

or piercing parts so that none adhere and all may be seen distinctly. The antennae should be separate and not overlapped. Roaches should be mounted dorsal (back) surface upward; ants either dorsal or lateral (side).

Once positioned, each part is then weighted down as may be necessary to prevent its curling up, adhesion, or other spoiling of the correct positioning. For this, use bits of broken cover glass, broken slides, particles of sawed or filed metal, short lengths of fine glass rod, or other convenient objects sufficiently small in size. Sometimes a tiny length of glass tube or rod can be wedged between two legs or two antennae so as to prop them apart. Once all is arranged, place a cover on the vessel and leave it undisturbed for 12 hr.; after this very cautiously pipette off the alcohol and replace it with 70A for a like period, using care not to disturb the insect. At the end of this period, the object should be hardened to the extent that future disturbances are not so likely; at the same time, it is rendered more brittle and hence increasing care should be exercised in subsequent handlings.

Go through the 82A and 95A for a few hours each in the same manner; then dehydrate as preferred and add the clearer. After an hour or so, work the specimen up onto a slide held beneath the clearer, with as little disturbance as possible. If the insect has wings, you can pick it up by one of them with a fine forceps; otherwise use a brush and, assisted by the liquid, swim the object to the center of the slide, flooding with the clearer to assist by floating. Align the specimen with the slide and make the final adjustments. The positions of parts cannot be changed, but slight disturbances can be corrected. Tilt the slide slowly to drain off excess clearer, then add balsam, cover glass props, and finally the cover. Put the balsam on beside, not on top of, the specimen (Fig. 44).

**Radula of Snail.**—In the head of a snail or slug there is a complex of muscles termed the “buccal mass” which oper-

ates a peculiar structure variously called the odontophore, radula, lingual ribbon, or "tongue," and which makes a very beautiful microscopic preparation. Not only is the detailed and complicated arrangement of the many "teeth" of the radula interesting to observe; they have value in classification, since the pattern differs in various species, and have been used also as test objects for lenses and for demonstration with polarized light. Snails rasp away vegetable fibers or other substances by means of this apparatus.

Cut off the head of a snail or slug and soak it for 1 or 2 days in 10% potash; this will corrode all materials except the radula. Pick this object out of the dish and wash it with several changes of tap water for a few hours. Place it upon a slide and lay a narrow strip of paper alongside each side of it. Add a second slide on top of the first and bind the two together at each end with thread or rubber bands, this compression being necessary to prevent subsequent curling. Handling the two slides as one, dehydrate and clear; then remove one slide and the paper strips, add cover glass props, and mount in balsam.

**An Isolation Mount.**—Secure a large grasshopper such as the Carolina locust (*Dissosteira carolina*), lubber grasshopper (*Rhombalea microptera*), or other available species. Any type will do, but the larger ones are easier to work with, especially at first. Kill by immersing in 70A, cut off the head, and transfer it to 95A for 3 hr., 100A for 1 hr., then either cedar or clove oil, 1 hr. Remove the head to a slide in a drop or two of the oil, preferably under a binocular, dissecting microscope, mounted hand lens, or any other form of low magnification at your disposal. With needles, dissect out the individual mouthparts. Figure 79 shows the completed slide with all parts properly oriented.

Remove each element separately to a clean slide and, one at a time, mount them so that all will be in their correct relative positions when the slide is completed. In mounting each one, apply a very tiny drop of thick balsam to the

slide at the exact spot selected, then press the part down into this droplet. Make any final adjustments necessary when all parts have been so mounted, add cover glass props, several drops of thinner balsam, and the final cover. The

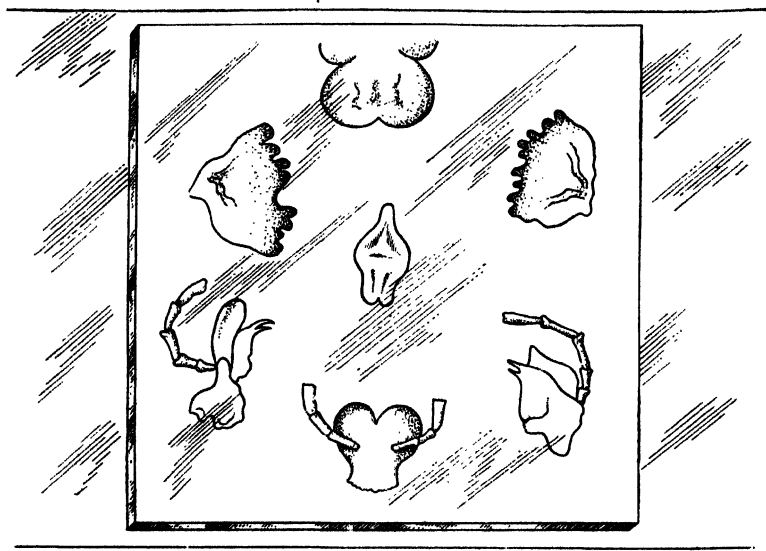


FIG. 79.—Arranged slide of grasshopper mouthparts.

thick drops are to assist in preventing shifting and the slide must be kept perfectly flat until it is absolutely dry and hard. If, after completion, any of the parts shift, a heated needle may be run in between the slide and the cover to rearrange. Heat melts the balsam in immediate contact with the needle and permits poking a part into place. Air bubbles may be pricked and removed by this same means.

Mouthparts of the cockroach and various beetles make excellent subjects of the same character. The proboscis of butterflies and moths, biting flies, blowflies, and honeybees are mounted whole, without such dissection. Biting flies, such as the horsefly, should have the parts teased as has been described for the mosquito, but left connected at the base. With the mosquito, a separate slide of the head may

be made, preferably one of each sex; it is customary to exhibit the whole head, with antennae, compound eyes, and the piercing-sucking type of mouthparts, the lancets well spread.

**Plant Materials.**—Quite a variety of botanical subjects lend themselves to the preparation of interesting whole mounts. Included are moss leaves, whole plants, fruits, spores, elaters; fern spore cases and prothallia; and, with higher plants, pieces of leaves, flower petals, whole flowers of such types as wheat, root hairs, epidermis of leaves, leaf hairs, pollen, seeds, and starches. On the whole, however, nearly all of these should be stained, mounted in glycerin, or otherwise processed by methods taken up in subsequent chapters; so most of them are deferred.

**Leaves.**—Small leaves, particularly those of the mosses, may be fixed for 24 hr. in formol-acetic-alcohol, washed for 2 hr. in running water, dehydrated, cleared, and mounted in balsam. Such slides are, however, decidedly inferior to glycerin mounts (Chapter 6) and show very little. Of more interest are various scales, hairs, and spines, often microscopic, found on the surfaces of such leaves as begonia, alyssum (hairs); goose grass, hop (hooked hairs); deutzia, mullein (stellate hairs); correa (scales); and nettle (stinging hairs). To secure these for whole mounts, the epidermis of the leaf is stripped off and mounted entire. One method is to use a fine scalpel and make a slight incision crosswise of the leaf, cutting through only the surface layer (epidermis); then work the scalpel in under this layer until loosened and finally grasp a free edge with fine forceps and pull off a strip. Fix, dehydrate, and mount in balsam as just described for whole leaves. Some workers prefer to rot the leaf in water first, to facilitate removal of the epidermis; still others take small squares cut from a whole leaf, boil them in nitric acid until separation of the epidermis begins, wash them well in water, and proceed as before. Many of these hairs

are good objects for the polarizing microscope; for this purpose they should be cut from the leaves and mounted flat.

**Stomata.**—Occasionally in the upper epidermis of a leaf and always in the lower epidermis, scattered all over the area of the leaf blade, occur pairs of bean-shaped *guard cells*, each surrounding a minute pore, the *stoma*. These connect air spaces within the leaf to the exterior, and are opened by the swelling and arching apart of the guard cells, closed by the shrinking and straightening of these cells. The factors of warmth, moisture, and light affect the guard cells so as to regulate the size of the stomata. So small are these openings that 2,000 or more are needed to equal the diameter of a pinhole. From 1 to 600 may be found in a single square millimeter of leaf surface.

These structures make most interesting microscopic objects when displayed as flat mounts of lower leaf epidermis, prepared as described on the preceding page. Usually such mounts are stained, but the foregoing outline is given here while we are on the subject of leaves.

**Raphides.**—These are crystals of calcium oxalate present in many plant tissues, as in the leaf epidermis of the onion, lily, and hyacinth. They are needle-like in shape and vary in size and groupings. Rhubarb is a good source of them; squeezing the juice from a cut piece of petiole—the stalk of the leaf—will yield specimens. With leaves, rot (macerate) pieces in water until thoroughly disintegrated, pipette off the water, and add fresh amounts, washing in this way repeatedly and allowing the raphides to settle to the bottom of the vessel between washings, continuing until no visible pieces of leaf tissue remain. Suck up raphides from the bottom with a pipette and place some on a slide, drain off the water, dehydrate at once in 100A, clear in xylene, and mount in balsam.

**Pollen Grains.**—Most of these appear to best advantage when stained, though the large and showy grains of hollyhock are also interesting in the unstained condition. Shake some pollen into a pillbox and allow it to dry thoroughly; then mount it in balsam or, better, place it in turpentine for several days, then in 70A for 6 hr.; 95A, several days; 100A, 1 hr.; clear in xylene, and mount in balsam. This complicated technique aids in getting rid of trapped air which is usually so bothersome with spiny pollen.

**Starch Grains.**—These bodies occur in seeds, bulbs, tubers, and other parts of a great many plants and may be obtained by grinding up the material in a mortar or by scraping with a knife on a cut surface. Scrape a raw potato or sweet potato; for wheat and rice, soak the kernels first, then grind. In either case, strain the resulting substance through a piece of fine muslin or linen, which will allow the starch grains to pass through but remove any coarse materials. Strain into a test tube of water, allow to stand to permit grains to settle, then decant. Repeat until the water is clear and no debris seen. Store in distilled water, in which starch is insoluble at ordinary temperatures.

One method of mounting is to place a drop of the water containing starch grains on a slide, set it aside to dry, and then mount in thin balsam. Another employs Mayer's albumen fixative, smearing this over the central portion of a slide, as is done to affix sections to slides (Chapter 13). When the fixative is almost dry, add a drop of the water containing starch grains and see that it spreads around evenly, using a toothpick, brush, or needle. Again, allow it to become nearly dry but, while still moist, put the slide into 70A, thereby coagulating the albumen. After 1 or 2 min., dehydrate, clear, and mount in balsam. Always for use with polarized light and usually for other purposes, starch grains are mounted unstained in this manner, though they are easily stained if desired.



## CHAPTER 6

### CELL MOUNTS

*In This Chapter:* slide-centering gauge; turntable, spinning cells, and affixing ready-made rings; flaming a cover glass; mounting in glycerin, glycerin jelly, and dry mounts in air; opaque and half-and-half mounts; mineral box mounts; Lazy Susan.

SOMETIMES materials cannot be mounted in balsam; at least they are preferably immersed in some other medium, often fluid or semifluid. Still other preparations are too thick for ordinary balsam methods. A few objects can be shown to best advantage as dry mounts, with air as the sole surrounding medium. All such cases require the manufacture of a *cell*, an unfortunate term since it is confused with an entirely different meaning of the same word, applied to the structural units of plants and animals. By "cell" in the sense used in this present chapter, we mean the space within a circular rim built up on top of the slide in order to provide a miniature aquarium or tank, as it were, in which to mount bulky, delicate, or opaque objects, in air, fluid, or thick balsam.

As there are a great many types of cells employed by microscopists, it will be just as well to experiment with nearly all of them in order to be able to exercise an intelligent choice among them. Cells are either formed with a liquid which is spun on and later hardened to a solid, or are made of rings of solid material cemented to the slide.

**Centering Gauge.**—For either type of cell it is essential to determine the exact center of the 3- by 1-in. blank slide on which the cell is to be built. This is done by the following steps: Take a 3- by 5-in. blank white card, such as is used in filing cabinets, or any similar rectangle of cardboard, and

on it place a slide, the long axis lengthwise of the card and approximately centered. Holding the slide firmly in place, trace around it with pen and ink, let dry, then remove the slide. With a straightedge, rule diagonal lines connecting the opposite corners of this diagram. Where these diagonals intersect marks the center of the rectangle. Replace the slide over this outline. Now the ruled lines show through the glass and indicate the center for purposes of mounting. Such a gauge is often useful with any kind of permanent preparation, whether or not cells are to be employed.

**Turntable.**—If cells are to be spun and the slides sealed, as proposed in this chapter, some form of turntable must be made or purchased.

A glance at the illustrations (Figs. 80, 81) will show the principle involved. The commercial turntable is of metal to give good bearing surfaces and a certain amount of



FIG. 80.—Commercial turntable.  
(Courtesy of F. Leitz, Inc.)

weight. The table proper is a circular disk of heavy metal pivoted in the exact center so as to spin easily in rotary fashion. One flip of the finger will make it spin for many revolutions. On some models a centering gauge is engraved

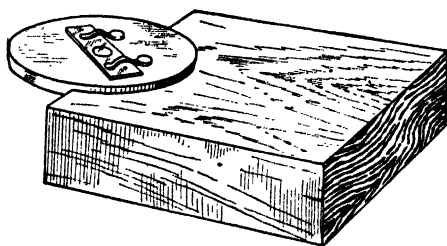


FIG. 81.—Homemade turntable.

and there are spring clips to hold a slide securely in position during the spinning operations. In the rear of this table is a flat hand rest, flush with the table, or else an elevated and removable hand rest.

Now it is obvious that if one takes a fine-pointed brush dipped in shellac and holds this to the slide at any point off center while the table is turning, a ring of the shellac will be spun upon the slide. The diameter of such a circle may be of any desired dimension; commercial models have ruled guide lines that coincide with the various sizes of circular cover glasses, the lines being purposely slightly smaller than the cover glasses so that these may completely overlap the spun rings.

In spinning cells one grasps the brush between the thumb and forefinger of the right hand, with the fist resting upon the stationary part of the turntable, then gives the wheel a spin with the left hand and carefully lowers the brush over the desired spot, leaving it in contact with the slide for a number of revolutions so as to build up a rim of the shellac. The slide is then placed in the evaporator to dry and later another ring is spun on top of the first. This is continued until a rim of the desired height is built up. When ready to mount any contents, the slide is warmed gently to soften the ring and drive off any moisture, the material is mounted, and then the cover glass is flamed (see opposite) and pressed carefully into place. Or, after warming the slide, an additional ring of thin shellac is spun before setting the cover glass. After drying, the slide is again placed on the turntable and one to several coats spun at the very edge of the cover glass to seal it on securely. When properly done, even an entirely fluid mount is rendered semipermanent. If such slides show leakage with the passage of time, it is always possible to add one or more fresh rings or to remove the cover glass, fill the cell again, and repeat the original operations.

The homemade turntable must meet the requirements of a perfectly circular wheel, accurately centered, so that it will spin without eccentric motion or wobbling; nothing else is of importance. The hand rest, materials used, size, and weight are all secondary. One having any ingenuity with tools can easily make his own turntable from the illustrations here provided.

We have referred to spinning shellac cells. A perfectly clear shellac does make a very good medium for this purpose; there are numerous others, however. Many workers prefer balsam itself and some like asphaltum varnish, the jet-black color of which is attractive and the material unexcelled in its cohesion and ease of manipulation. If balsam is used, it should be thinner than that ordinarily employed as a mountant so that it will flow readily. Gold size is another widely used cell maker. The writer prefers any of the better brands of enamels and lacquers, especially for sealing finished slides, since they add the touch of lively color, such as jade green or Chinese red. Bell's cement is a well-known ring finisher, commercially available.

**Flaming a Cover Glass.**—Not only in the present case of cell making but in many other instances—perhaps for all slides—it is a wise precaution to *flame* the cover glass before mounting, ensuring that all moisture has been driven off. Grasp the cleaned cover near one edge with a forceps and pass it with medium speed through the tip of a flame, as that of an alcohol lamp or bunsen burner, first one side of the cover and then the other, alternated, twice each. The speed to be used can soon be learned since, if the motion is too slow, the cover will crack in the flame.

Circular cover glasses are used well-nigh exclusively in making cells since square covers cannot be ringed on a turntable. Slides on which cells are to be spun or affixed must be scrupulously clean, otherwise varnishes will not adhere. Use the glass-cleaning mixture when getting slides ready for this work.

**Ready-made Cells.**—Instead of spinning cells, one may cement on any circular ring of suitable diameter. Gummed cloth reinforcing patches are commonly sold at stationers for placing around the holes of notebook paper used in ring binders; one of these makes a very shallow cell and several of them superimposed will provide a deeper one. By using

cork borers, gun punches, or other circular metal punches of varying sizes, one may stamp circular disks from cardboard and use one or more of these in making cells. Choose two punches, one for the outside, the other for the inside

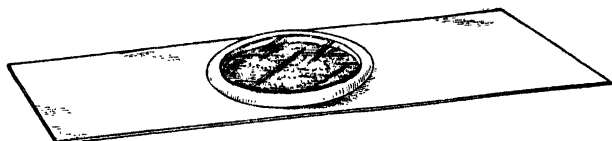


FIG. 82.—Cell mount made with bone embroidery ring.

diameter, differing by about  $\frac{1}{8}$  in., to provide a cell of proper width. All paper and cloth cells, when completed, should be sized on the turntable with shellac or lacquer so that no leakage can occur.

Even better cells are made by punching disks from sheets of cellulose acetate, now obtainable in many weights. By choosing a sheet of suitable thickness, a cell of required depth is obtained at a single operation. For many years, workers have also employed one or more of the following as cell-making rings: bone embroidery rings, brass curtain rings, glass, hard rubber, aluminum, and still other rings, with plastic materials coming in more recently (Fig. 82). All such rings should be accurately centered on very clean slides, then cemented in place by using duPont's household or other modern cement. Aluminum rings ought to be polished, with both upper and lower surfaces bright, clean, and perfectly flat.

It is suggested that for the following exercises you prepare in advance a number of cells, some spun, others ready-made, and of varying materials, diameters, and depths. A certain amount of practice is needed to gain skill in this work and every microscopist should be able to turn out professional-looking cells quickly and with ease. As some time is required for drying at the several stages of cell manufacture, it is well to have a quantity on hand, held in reserve for mounting needs. Keep in mind, too, while we are discussing mountants, that most materials may be placed in balsam

within cells. It is frequently a mere matter of preference whether one wishes to use a thick layer of balsam along with cover glass props, a cell, or a depression slide in balsam mounts of the bulkier objects, such as a thick insect leg.

**Mounting in Glycerin.**—Glycerin, also spelled glycerine, provides an excellent fluid mountant which will preserve objects, yet remain perfectly clear and transparent. Its use does not involve heat, strong alcohols, xylene, or other reagents that might prove harmful in special cases or with delicate materials. The aphid previously described as a balsam mount can be prepared even more perfectly by the glycerin method. It might be as well for you to use this same subject for the present experiment in order to compare the two.

Since glycerin is miscible with water, the specimen should go into it from water. Kill an aphid with a cyanide jar and place it in water until saturated. Select a cell of appropriate depth, no deeper than the specimen requires to be held in place without crushing, and spin a fresh ring of varnish on top of the cell rim. As soon as the surface of this coat is tacky, breathe in the cell to moisten it so that the glycerin will make contact throughout and no air bubbles form. Fill the cell flush to the top with glycerin and put in the aphid, positioning it with needles; then breathe on a cover glass and apply this moistened surface to the cell, pressing the glass down gently all around with the butt end of a needle handle, a pencil, or other convenient nonmetal implement. See that the cover adheres firmly all around.

It is now most important to wipe off, carefully and completely, all excess glycerin that may have exuded when the glass was pressed into place. Do this with cotton or a bit of soft old rag, wrapped around the forceps tips, a needle, or toothpick, and see to it that the job is complete. As soon as the edge of the cover glass around the rim of the cell is perfectly dry, spin on a finishing ring and later, after this dries, another. The various cements and lacquers will not

adhere to the glass if there is any trace of moisture; on the other hand, if too long a time elapses before the first coating is spun, air bubbles will work in under the cover as the glycerin evaporates.

Whenever it is necessary to fix an object permanently in place in a glycerin mount or to center the object, some accessory devices will prove helpful. Ordinarily the tension of the cover on the specimen should be just enough to hold it without any distortion or crushing, but with rather heavy materials there is apt to be drifting. To avoid this, before filling the cell, place a small spot of gum tragacanth on the bottom center of the cell, breathe on it, and apply the specimen. Allow it to harden for a few minutes, then fill the cell with glycerin, and continue as before. To center an object, as the head of a bee or a fly, thread a fine needle with a hair and sew this through the head. Grasping an end of the hair with each hand, lower the object into the cell and imbed the two hair ends in the shellac on opposite sides of the rim. Arrange the aspect wanted by twirling the hair ends; center by pulling gently on one end or the other. When the cover glass is affixed but before final sealing, cut off the protruding hair ends with fine scissors.

**Mounting in Glycerin Jelly.**—A great many technicians prefer this mountant to glycerin since it sets as a solid at room temperatures and hence holds objects more firmly in place as well as making a more permanent preparation. Before mounting, the container of glycerin jelly is placed in a vessel of warm water until melted. Then proceed exactly as for mounting in glycerin, including the sealing of the finished slide. Avoid overheating or prolonged heating of the jelly at any time, as this changes it to a form that will not remain a solid at ordinary temperatures.

**Two Dry Mounts.**—As may have occurred to the reader, a cell permits imprisonment in nothing more than the air

within the cell, providing the object is perfectly dry and will not deteriorate. For example, take a small quantity of ocean beach sand and, placing it in a fine strainer, wash it thoroughly under a water faucet so that all clay, dust, and other foreign matter is rinsed away and the sand left perfectly clean. Dry completely on the slide warmer or over any gentle heat, covering to exclude dust. Take up as much of this sand as will remain on the tip of a small pocket-knife blade and place it loose within a shallow cell. Flame a cover and seal it on. When dry, this makes an attractive mount inasmuch as the sand is free to move about and tilting or jarring the slide will expose various aspects of different grains. The quantity of sand should be such as nearly to cover the floor of the cell, but without overlapping or piling up.

Secure a butterfly or moth and kill it by decapitating or in the cyanide jar, or merely by pinching the thorax lightly between the thumb and forefinger to asphyxiate the insect, which is released when its wings cease to quiver. Do not touch the wings with the fingers at any time. Clip off a wing and put it away to dry for 24 hr., then gently scrape off the fine dust-like scales with which the wing is clothed and allow them to fall into a shallow cell. A knife blade held vertically to the plane of the wing makes a good scraper. Warm the slide over an alcohol lamp to dry the scales as well as to soften the cell rim; then flame a cover glass and press it upon the tacky rim. Seal when dry. Wing scales may also be dehydrated, cleared, and mounted in balsam, with or without a cell.

**An Opaque Cell Mount.**—In all cases where objects are too thick or dense to be observed by transmitted light, an opaque mount is in order and, if sufficiently bulky, within a cell. In some instances it would be advantageous to look at an object first by transmitted, then by reflected light, regulated by means of the mirror, and even to use both lights together on occasion. For materials such as clay soil



smears, fingerprints, protozoa with shells, textiles, and papers, mounts may be made on clear glass, as we have been doing; if, when examined by reflected light, there seems to be too much light getting in from below, place a piece of black photomount paper or cardboard on the stage under the slide. This will provide an excellent, lightproof background.

On the other hand there are some objects that cannot at any time be viewed to advantage by transmitted, but only by reflected light; here it is well to provide the slide itself with an opaque background. We have seen slides with the reverse side painted with black lacquer or asphaltum varnish under the object, to exclude light. Such slides will not lie perfectly flat on the stage if the coating is thick enough to function properly and do not present a very neat appearance; hence we prefer painting the inside bottom of the cell. Another method is to cut out a circle of black paper, lightweight photomount preferred, of the exact diameter to fit the bottom of the cell, and cement this in so as to lie smooth and flat. Or a black paper circle of the same diameter as the cell to be spun or affixed may be fastened to a blank slide in the first place, and the cell built on top of this (Fig. 82).

Since the writer has found the last method best, the various steps will be reviewed here so that the technique will be perfectly clear, using as a subject this time a small portion of oölitic sand or white dune sand. Clean a blank slide very thoroughly and flame to dry completely. Over the centering gauge, affix a circle of black photomount paper of the same diameter as that of a bone embroidery ring, secured at the notion counter of a department store. A ring of approximately  $\frac{3}{4}$  in. is suggested. To affix the paper, coat one surface with duPont's household cement and let it dry until tacky; then press this down on the center of the slide and place it away to dry with a weight on the paper. Next day, rub both surfaces of the ring on fine emery paper to flatten and polish; rinse, wipe clean, and dry over low heat.

Cover one surface with the cement, again letting this become tacky, then press it down firmly over the paper circle, taking care to center it exactly. Again place it away to dry, weighted. Next day, put a layer of the cement on the upper rim of the ring, let it become tacky while adding the proper quantity of well-washed sand, flame a cover glass of the correct diameter, and press this down into the cement. Carefully wipe off any excess cement and place the slide away to dry, weighted. Here care must be used not to employ a weight that would crack the cover; nuts, washers, and bullets are suitable. On the following day, place the slide on the turntable and seal with lacquer of any desired color; repeat with a second coat when the first is dry. Polish off any fingerprints and add the permanent label. For black sands use a white paper background.

**Half-and-half Slide.**—By the same method it is perfectly possible to cover only half of the cell bottom, using a semi-circle of black paper, and thus prepare a cell in which half of the material may be viewed by transmitted light, the other half by reflected light. A good subject for this sort of mount is a quantity of the shells of foraminifera (Fig. 100), either recent or fossil, further description of which appears in Chapter 10.

The shells may be placed loosely within a cell, as was done with sand, but such a slide is apt to be perishable. Jostling of the shells against one another will injure them and flake off a fine powder which will cloud the under surface of the cover glass. A much better mount is made by painting the bottom of the cell, the half covered with black paper as well as the other half of clear glass, with a thin coating of balsam—enough so that the shells may be spotted about at intervals like so many flies on a sheet of flypaper, holding them securely in place when dry. Add the cover glass only after thorough drying.

An alternative method is to coat the bottom with gum tragacanth and spot the shells around on this, breathing on

the gum to set the specimens. Dry and then cover. If preferred, the whole cell may be filled with balsam after the shells are well anchored in place. If not affixed in this manner in a balsam mount, they will likely all drift together along one edge of the cover, ruining the preparation.

**Deep-cell Crystal Mount.**—Construct a high cell from a glass or bone ring, with a black paper bottom, and in it affix by a spot of balsam a small group of mineral crystals of some showy type. Do not fill the cell with any mountant. Cover and seal when dry. Recommended subjects are azurite, malachite, sunstone, and vanadinite. When viewed by low power in strong reflected light, such a mount is a most gorgeous sight.

**Box Mount of Crystals.**—Another very effective way of displaying groups of showy mineral crystals dispenses with a slide altogether. Secure a number of small cubical pasteboard pillboxes from your druggist. Remove the lid and paint or stain the interior of the bottom half of the box a dead black. When dry, cement a small truncated pyramid of cork to the center of the bottom half in such a way that the apex of the pyramid reaches about halfway up from the box bottom. On this cork, cement the group of mineral crystals, using duPont's cement or balsam. The name of the specimen is written on the lid and a serial number on both the lid and the bottom half so that lids cannot become mixed. Keep the lid on when not in use, to exclude dust. The box may be placed on a slide or directly on the microscope stage for observation. Here again a very beautiful sight is presented with the proper vertical illumination.

**Lazy Susan.**—We are indebted to Mr. George L. English, well-known mineralogist, for this idea, shown in Fig. 83. He places the microscope lamp in the center of the small revolving table, known as a "Lazy Susan" and formerly

much in vogue on the dining table for dispensing condiments. The microscope is near the edge of the table, but no matter how the Susan is revolved, the relations of microscope and lamp remain the same and ensure correct lighting.

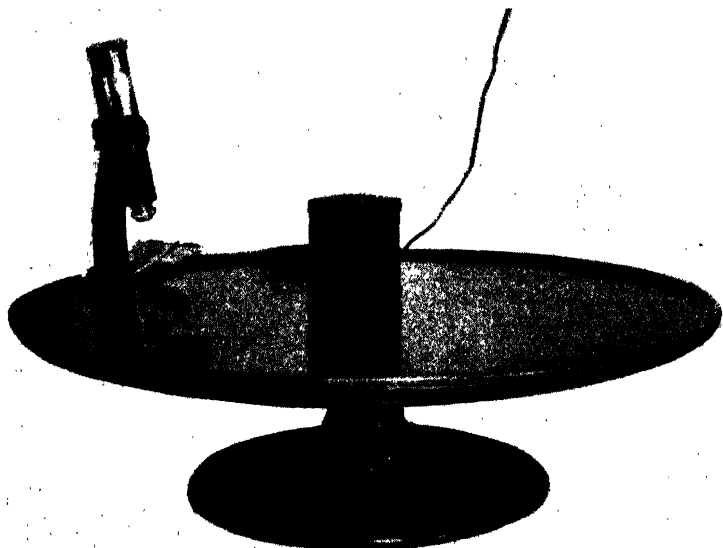


FIG. 83.—A Lazy Susan put to work with a Bausch & Lomb Gem microscope and substage lamp.

A number of people may seat themselves around a small table, preferably a circular one, on which the Lazy Susan and its contained instruments are placed. The operator puts a box mineral mount (or slide of any description, for that matter) under the microscope, focuses, and adjusts the illumination, using a substage lamp for transparent mounts, a gooseneck student lamp for opaque ones. When all is properly set up, he swings the Susan to the right so that the microscope comes under the eye of the person at his right, and so on around, making a few remarks meanwhile as to what is to be seen. For a small audience this makes a most effective way of exhibiting one's slides; if a regular sequence

of related slides and remarks about them is planned in advance, one can provide a most entertaining evening for a group of friends. We have studied Mr. English's splendid collection of mineral box mounts in this manner, but the idea is equally adaptable for any type of slide or for living organisms in watch glasses.

## CHAPTER 7

### STAINED WHOLE MOUNTS

*In This Chapter:* slides of insect anatomy, hydra, hydroids, worms; botanical preparations; histologic whole mounts; preparation of fixing and staining solutions; washing in running water; narcotizing methods.

AS WAS explained in Chapter 4, most whole mounts are single stained by the regressive method; a few may be double stained. Whereas most research involving the microscope employs thin sections, the general amateur, protozoologist, helminthologist (specialist in the worm groups), and entomologist deal more particularly with toto staining of whole mounts; the present chapter is designed to furnish material for practice in this somewhat difficult and exacting technique. He who can master the art of making fine whole mounts can hold up his head among the most exalted company of microscopists; for many a noted cytologist and expert with chromosomes has never mounted a planaria and knows nothing of the many problems presented by the lowly hookworm.

#### EXAMPLES

**Honeybee Sting.**—This is an excellent type of mount with which to begin—more or less of a classic among whole mounts and a little more troublesome than the slides thus far considered, but by no means beyond the powers of the merest beginner if he is capable of taking pains and acquiring precision and dexterity in his manipulations. The first slide is apt to leave something to be desired; but try again. Perfection is just around the corner!

Capture and kill several honeybee workers in a cyanide jar and snip off their heads with scissors. Place them to soak

in a small covered dish of tap water for several days—usually 48 hr. is adequate—until the odor becomes somewhat offensive. Remove a bee, place it on the work table on its back and with the left forefinger press gently on the

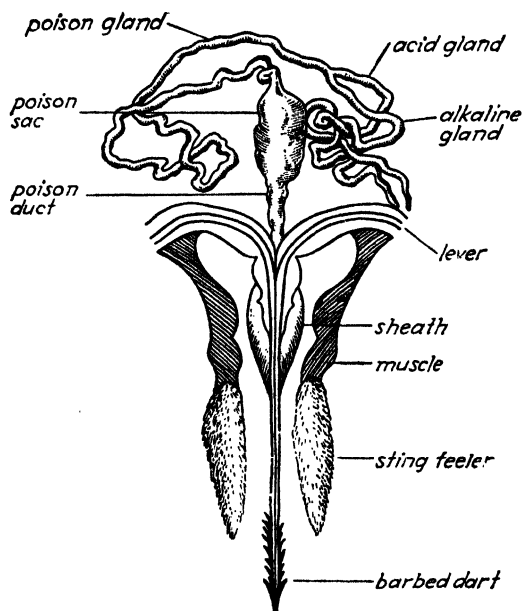


FIG. 84.—Stinging apparatus of the honeybee.

abdomen. This should protrude the sting which is then grasped by a fine forceps held in the right hand and, by pulling carefully on the sting while pressing on the abdomen and squeezing lightly from the head end toward the sting, the whole stinging apparatus is usually expelled intact. Remove it for examination to a watch glass or small stender of fresh water, rinsing as may be necessary.

The apparatus (Fig. 84) consists of two barbed darts enclosed in a sheath, operated by muscles attached to chitinous levers, a pair of finger-like sting feelers, and, anterior to the sheath, a duct leading to a large poison sac, from which in turn issues a single tube, the poison gland. Some distance forward, this divides into two tubes, the

acid gland and the alkaline gland, which end blindly. Inspect your specimen with a hand lens and, if not complete, reject it, and proceed with another bee.

Once a perfect specimen has been obtained, pass a needle along the sting sheath from base to apex to engage the barbs on the two darts so as to pull them out and expose them, if not already fully visible. Also prick the poison sac with the needle in several places to admit reagents freely and avoid trapping air within this bag during mounting. Fix in 10% formalin for 1 hr., rinse in several changes of 50A during 15 min., then remove to a slide by swimming, assisted by a brush and pipetting. Formalin fixed material should not be washed with water.

The whole apparatus is now stained to render all parts more conspicuous. For this purpose either borax carmine, eosin, or fast green is excellent. (See the last part of this chapter and also Chapter 17 for their preparation and use.) If borax carmine has been chosen, stain for 1 hr., then rinse by flushing with a pipetteful of water, several times, until no further stain is extracted, and drop on 50A. After a few minutes suck this up and replace it with 70% acid alcohol and decolorize until the stain ceases to come away freely and the object is brilliant in tint but not so heavily colored as to obscure detail. The staining should be bright and at the same time thin by transmitted light under the microscope. Wash in fresh (pure) 70A to stop the destaining action, complete the dehydration, clear, arrange the parts carefully on the slide in their correct topographic relations, and mount in balsam with cover glass props.

If any acid is carried over into the balsam, the stain will fade in time; for this reason many workers prefer to follow the destain with 70% alkaline alcohol rather than straight 70A, ensuring a neutralization of any remaining acid. Also many technicians advocate the *compression method* with this and many similar items, done as follows: When the object is in the 70A or 70% alkaline alcohol, arrange the parts as desired in the final mount, then add a second slide



on top of that containing the specimen, and tie the two together at each end with thread. Immerse these two slides in 82A for 10 min., then in 95A for several hours. Cut the threads and very gingerly remove the top slide, flooding with the alcohol if necessary to prevent adhesions, then proceed as before. This method makes a very flat mount and excludes air bubbles. Instead of thread, the two ends of the combined slides may be held together with rubber bands, with spring-clip clothespins, or by means of a spring compressor often used to weight down the cover glass on a thick mount and easily bent into shape from brass wire.

**Other Preparations of Insect Anatomy.**—The salivary glands of certain insects make an interesting subject of a nature similar to the bee sting in preparation. Kill a cricket or cockroach and soak it in water until an unpleasant odor is noted; then pull off the head, whereupon the esophagus, salivary glands, and crop usually come away with the head. Fix, stain, and mount as for the bee sting, trying out a different stain this time.

The gizzard of a cricket provides an excellent whole mount. It generally comes off with the head, as per the directions for salivary glands, and is then slit open lengthwise, rinsed out, fixed, and mounted unstained with the inside lining exposed.

With a bit of skill gained from practice, it is not difficult to dissect out the entire alimentary canal or the nervous system from such insects as grasshoppers, roaches, crickets, and bees, especially if you have any form of optical aid such as a dissecting microscope. When fixed, stained, and spread out in proper fashion, these subjects are completed to mounting in balsam and placed under large rectangular cover glasses, of a size indicated by the dimensions of the material, and provide whole mounts of which any technician may well be proud.

**Hydra.**—A fine subject with which to carry further the art of making whole mounts is the ever-popular hydra. There are a number of species, the brown hydra being recommended for this work and usually the most abundant in



FIG. 85.—Typical habitat of hydra and planaria.

nature. Collect these animals from the undersides of lily pads, from the stems of all sorts of aquatic vegetation, or from dead leaves on the bottoms of clear, sunlit pools (Fig. 85). They resemble small threads, frayed at the upper end, and range in length from  $\frac{1}{8}$  up to nearly 1 in., according to whether contracted or expanded. If a few have been placed in a battery jar or rectangular aquarium in which water plants are growing, there will be many present after a while, providing a continuous supply. Remove hydras by scraping the sides of the glass vessel with a razor blade or by sucking them into a pipette, immediately squirting out again into a syracuse watch glass. If specimens remain for more than an instant within the pipette, they will adhere and then are quite difficult to remove.

After a hydra has been placed in a watch glass, draw off with a pipette nearly all of the water, leaving the animal

just enough in which to expand fully. Have ready some hot Bouin's fluid, the fixer to be used in this case. Watch the hydra. When he has fully expanded, squirt a pipetteful of the hot fluid over him from base to apex, sweeping the tentacles out beyond the animal and fixing him instantly so that he has no time to contract. The tentacles occupy the apex end, and only well-expanded specimens are worth mounting. Fill the watch glass with the hot Bouin's fluid and allow it to act for 30 min., then wash well in several changes of 70A for not less than 10 min. Note that in the case of Bouin's as a fixer, alcohol is the wash instead of water, the reagent being an alcoholic solution. Stains to be used should be alcoholic also by preference, though aqueous dyes may be employed should there be any advantage in so doing.

Hydras are best stained with hematoxylin, borax carmine, or fast green. To gain experience in the use of different stains, let us select the first named. Dilute a small quantity of Delafield's hematoxylin with an equal amount of distilled water and apply in either the watch glass or a small shell vial. For the latter method, pipette off most of the washing alcohol and pour the remainder, with the specimen, into the vial; then pipette off the last of the alcohol and replace with stain. Put away to overstain for 24 hr.; then pour the hydra into a watch glass or small stender again, pipette off the stain, and cover the animal with 70% acid alcohol which is left until color ceases to come away. Wash in 70% alkaline alcohol, then straight 70A, and go on to dehydrate, clear, and mount in balsam with low cover glass props. Many other animals may be processed by this method.

Bouin's is an excellent general fixer for invertebrates and for sections of organs; it is almost impossible to overfix with this reagent.

**Hydroids.**—This is a collective term designating hydra-like animals, nearly all marine, that reproduce by an alternation of generations. One of these generations is the *hydroid*

(like a hydra) and the individual animals are known as *polyps*. The whole *colony* is *sessile*, that is, attached to some object in the shallow waters near shore, such as rocks, shells, wharves, piling, and submerged vegetation, and each polyp rises by a side branch from a common stem, in plant-like fashion. For years the earlier naturalists thought that these colonial coelenterates were plants, but their animal nature was clearly established when life histories were worked out and it was found that certain specialized polyps on mature colonies give rise to *medusa buds*, which are set free and become the familiar jellyfish (*medusa*) type of animal. Every stage of organization is shown in the life histories of various species; in some the polyp type predominates and the medusa is reduced, in others the medusa is much the more prominent generation, and in still others they are about equally developed. Hydra itself represents a type in which the medusoid stage has been dropped out altogether. The better known genera include Obelia, Sertularia, Tubularia, Pennaria, Plumularia, Bougainvillea, Clava, and Hydractinia, the last-named found growing on the shells of hermit crabs. All of these make fascinating subjects for observation under the microscope while alive, as well as most interesting whole mounts.

Collect hydroids at suitable places along the seacoast by scraping them from submerged objects and study them in their natural condition in a syracuse watch glass or biological finger bowl containing sea water. For slide making they are killed by gradually narcotizing so that they will die in a fully expanded position. Some technicians use chloral hydrate (*Caution*: very dangerous internal poison) or formic acid for this purpose, but ordinary epsom salts (magnesium sulphate) will serve as well as anything. Add small quantities from time to time, over a period of about 12 hr., to the sea water in which the specimens are kept. When they are completely expanded and no longer respond to the touch of a needle, fix by pouring off the water and replacing with corrosive-acetic for 12 hr. or overnight; then wash in run-

ning water for a like period, as described in the last part of this chapter.

It is important to know and remember that all fixers containing mercuric chloride must be treated with iodinated alcohol during dehydration; otherwise the object will contain innumerable black "pins" of mercuric chloride crystals. Iodine reacts with this salt to form the more soluble mercuric iodide, which is then washed out. In practice the iodine is added to the 70A, enough to give a port-wine color; if the reaction removes this color, and as often as the color is removed, more iodinated 70A is used until no more decolorization occurs. Then the object is placed in fresh 70A; this in turn must be renewed as often as it is discolored by extracting iodine. Usually one change of each reagent is all that is necessary.

With the hydroid specimens washed, place them in 50A for 30 min., then in iodinated 70A for 6 hr. If decolorization takes place during this time, pour off the alcohol and replace it with fresh iodinated 70A, continuing until the port-wine color persists. Then change to pure 70A for 6 hr. more, or overnight, changing once or twice if the alcohol is noticeably colored by the extraction of the iodine. Now pass to 82A for 15 min., and then 95A for a few minutes. Pipette off this alcohol and cover the specimens with fast green in 95A for 1 min.; rinse in fresh 95A and examine to see if the amount of staining is correct. Use acid 95A to destain, if necessary; apply the green for a longer period if understained. Only trial and experience can prescribe the exact time for this staining, but fast green works very rapidly. A good slide will show an elegant clear palish green.

When the material has been satisfactorily stained, dehydrate rapidly, clear, and mount in balsam. Clove oil is recommended by some, cedar oil by others as a clearer, but good results may also be obtained with xylene.

**Planaria.**—A schedule for mounting this flatworm has been given in Chapter 4, but a few additional remarks will be

found helpful. Refer to a text of general or invertebrate zoology for details of the classification, appearance, habits, and habitat (Fig. 85) of planarians as well as flukes and tapeworms. Full-grown planarians average around  $\frac{1}{2}$  in. in length when extended, are very flat and ribbon-like, and are dark grayish or brownish red in color. They may be collected on the undersides of logs and stones in brooks and ponds and, if wanted in numbers, are easily procured by baiting. Fasten a small piece of red meat to the end of a string and lower this into a brook, near shore. After 1 or 2 hr., if planarians are present, there will usually be many attached to the meat when it is hauled in. Compressing a specimen during fixation is advisable as otherwise it will curl.

Flukes are internal parasites of vertebrates, the sheep liver fluke (*Fasciola hepatica*) having long been a standard example of the class Trematoda in zoological courses. It is a European species, but has become naturalized in parts of the United States. Specimens for whole mounts are best procured ready fixed from a supply house; if one is not particular as to species, there are many easily secured types that parasitize our wild and domestic animals, the common leopard frog providing a number of forms that make most attractive and worth-while mounts. Kill a frog and remove the lungs, urinary bladder, and rectum to separate watch glasses containing normal saline solution. In each case, tear and tease the organ apart with needles; if flukes are present, they are readily visible without magnification and can be processed as outlined for planaria. Corrosive sublimate is an excellent fixer for flukes; Mayer's paracarmine and also his hemalum are widely used as stains.

**Tapeworm Composite Mount.**—Some knowledge of the anatomy and life history of these, probably the most interesting of all internal parasites, should be gained by reading before attempts at whole mounts are made. Specimens can be secured at slaughterhouses from food animals; but, as the best material comes from dogs, you will need to enlist the

assistance of a veterinary or, even better if available, some person connected with a city pound who will permit you to dissect dogs that have been dispatched. Chance captures of rats and mice around the house will also provide good hunting along the course of the intestine; and most cats are likewise infected.

Slit open the intestine and remove any worms to a vessel of warm normal saline, using the opportunity to watch the interesting animals under magnification while still alive. When ready to process, there are several killing methods recommended by various authors to fix a specimen in the extended state—they are obstinate in this respect. Barker wraps the living worm around a blank slide and immerses this in the killing agent, removing it as soon as dead. Corrosive-acetic, 1% chromic acid in water, and formol-acetic-alcohol are all good reagents for this purpose. Chromic acid produces excellent expansion of the worm but will also cause curling, so the object should be removed, as soon as completely relaxed and killed, to Bouin's or either of the other two killing agents already mentioned. Coatney flushes the worms from the host intestine by means of a hose connected to the cold-water tap and then immerses them in ice water for 1 to 4 hr. until they are thoroughly relaxed. Specimens are then transferred to formol-acetic-alcohol for 24 hr.

As a stain for tapeworms, Delafield's hematoxylin, borax carmine and alum cochineal are all excellent, but in order to introduce a new technique, let us try out Mayer's hemalum (Cort's method), along with a different fixer as well.

Kill a tapeworm in 1% chromic acid and, as soon as it has expanded and appears lifeless, cut off the scolex (so-called "head") with about  $\frac{1}{2}$  in. of the "neck" region. Divide the rest of the worm roughly into thirds and from the middle of each third take one or two  $\frac{3}{4}$ -in. lengths. The anterior third will contain immature proglottids (segments), the middle third mature ones, the posterior third gravid ones. This gives four parts—scolex, immature, mature, and gravid proglottids, all of which are to be mounted on a single slide,

as shown by Fig. 86. These can be processed in four separate small stenders if desired, though since they are readily told apart by size and shape, they may as well all be done together.



FIG. 86.—Photomicrograph of composite slide mount of tapeworm, *Taenia pisiformis*, (5.1 X). Left to right, scolex, immature, mature, and gravid proglottids.

Place the pieces, as soon as severed, in formol-acetic-alcohol, a famous formula generally termed FAA. It will fix well in 24 to 48 hr. but has the advantage that material may be left in it indefinitely as it is almost impossible to overfix with this reagent. Inasmuch as the fixing fluid is largely 50A, no preliminary washing in water is necessary and the pieces are rinsed in 50A and then placed in Mayer's hemalum for 24 hr., until considerably overstained; then they are very quickly and thoroughly destained in a strong (2 to 4%) acid 70A until the color looks well under the microscope and all internal structures stand out brilliantly. Stop the destaining in fresh 70A and proceed with the usual dehydration and clearing. Refer to Fig. 86 for details of mounting and use cover glass props.



Never attempt to compress the scolex, which would be ruined thereby; some or all of the proglottids may need this attention. Some microscopists prefer to compress only those segments that show a tendency to curl; others compress them all as a matter of routine. It has also been recommended that segments be bound to a piece of bristol board or other stiff cardboard by wrappings of thread, the piece then immersed in reagents.

**Roundworms.**—Under this classification belong many dangerous parasites of great interest. We shall confine remarks here to hookworms, the type most suitable for whole mounts. Balsam mounts are possible and perhaps the majority turn out well, but such specimens have a strong tendency to turn black when transferred to balsam from the clearer, and are rendered opaque and unfit for use. If plenty of material is available and but one or a few finished slides desired, it might be as well to follow the technique given for planaria and simply throw away all slides that go bad. Several alternatives have been proposed, however, and for more certain results we suggest you try one or two.

Glycerin-jelly mounts for many planarians and nematodes (roundworms) are preferred by many technicians, the optical properties being superior to balsam mounts for one thing. Since these are apt to be less permanent, however, one form or another of a combination of the two methods has been tried, with excellent results. With a glass-writing pencil of the soft wax variety, draw a  $\frac{1}{2}$ -in. circle on a clean slide, using the centering gauge. Bear down with some firmness in order to leave a heavy deposit of the wax on the slide. Within this shallow cell mount a hookworm in glycerin jelly and place it in the evaporator to dry. Later, surround the wax cell with balsam of an amount and distribution so it will fill up all space outside the circle but within the limits of a large ( $\frac{7}{8}$  in.) square cover glass. This effectively seals the jelly against evaporation and leakage.

Becker and Roudabush advocate placing the worms in a

vial half filled with normal saline and shaking them vigorously for 3 min. to clean as well as fatigue them; they treat both flukes and nematodes in this manner. Pour off the saline and fix the specimens in hot 70A, 96 parts, and glycerin, 4 parts. Pour into an evaporating dish or wide-mouthed bottle and keep on top of a warm paraffin oven or incubator for slow evaporation of the alcohol until the worms are concentrated in almost pure glycerin. Proceed as before from this point. Borax carmine may be used as a stain, but the experience of most workers has led them to the belief that unstained specimens are best in this group.

The same authors present an interesting modification of balsam mounting for nematodes. After the shaking in salt solution, kill the worms in hot 70A and pass through 82A, 95A, and 100A,  $\frac{1}{2}$  hr. in each, ether-alcohol, 1 hr.; then transfer to a petri dish and cover with thin celloidin, orienting the specimens so that they do not touch one another. Cover the dish to permit slow concentration of the celloidin. Occasional removal of the lid will hasten the process and does no apparent harm. When the celloidin has become firm, flood with 82A for 30 min., then cut small squares of the celloidin, each containing a worm. Pass each square to 95A, 30 min., and clear in beechwood creosote, 30 min.; then mount on a slide in balsam. Because of the block of celloidin, the cover glass will need no props. (See Chapter 13 for preparation of celloidin.)

**Botanical Preparations.**—A piece of leaf epidermis to show cells and stomata may be secured and processed as outlined in Chapter 5, but with the addition of staining. Delafield's hematoxylin can be used on fixed material, after washing in water. Stain for 30 min.; then rinse again in water, dehydrate, and, when in 95A, apply alcoholic eosin for 1 min.; rinse in fresh 95A, complete the dehydration, clear, and mount in balsam. Compression or patient unrolling will overcome any tendency to curl up.

Fern prothallia make most desirable whole mounts. They

may be collected from the pots and shelves of greenhouses where ferns are grown, from rotting logs or damp, shaded hillsides or in bogs; they may also be grown from spores. Fix in chrom-acetic 24 hr., wash in running water 6 hr., stain in Delafield 30 min., rinse, destain slightly when in 70A, thence through alkaline 70A to complete dehydration, and finish as usual. Chamberlain prefers a much more careful and exacting routine. Fix, wash, and stain as above, then wash in water slightly acidulated with hydrochloric acid for a few seconds, followed by a thorough washing in tap water. Dehydrate through a very gradual series of alcohols, 2 hr. each, consisting of 2½, 5, 7½, 10, 20, 35, 50, 70, 82, 95, and 100 percentages of alcohol. Leave in the 82A for 24 hr. to harden. Then use mixtures of increasing strengths of 100A and xylene, beginning with 5 xylene to 95 parts of 100A, followed by 10, 20, 35, 50, and 75 parts of xylene, thence to pure xylene, 1 hr. each. Transfer to a mixture of 10 parts of xylene to 1 of balsam which is left uncovered until most of the xylene has evaporated, concentrating the balsam, in which the prothallium is then mounted on a slide. This extreme care to graduate all of the steps prevents shrinking and plasmolysis and secures a much finer preparation. A counterstain is unnecessary but can be used; fast green or Lyons blue gives pleasing results.

Algae and pollen grains are deferred for treatment to Chapter 8.

An interesting whole mount that adds another step to our series of procedures is a stained preparation of a macerated leaf skeleton. Maceration is a form of rotting whereby certain elements are softened and removed, and others, more resistant, remain unharmed. By such a method, the "skeleton" of a leaf, that is, the branching system of fibrovascular bundles that carry materials to and from the leaf and at the same time provide it with a stiffened framework, may be isolated and displayed apart from other tissues. The bundles are thereby rendered much more conspicuous.

In a leaflet published by the Turttox Service Department

of the General Biological Supply House, the following steps are recommended: Boil the leaves for 1 hr. in a macerating fluid consisting of sodium carbonate, 4 oz.; calcium hydrate, 2 oz.; and tap water, 16 oz. To prepare this reagent, boil the chemicals in the water for 15 min., cool, then filter. As the leaves are boiled in this mixture, replace any water lost by evaporation.

At the end of the hour rub a leaf gently; if the soft tissues do not come away readily, boil 15 min. longer. For rubbing, lay the leaf in the bottom of a flat dish, cover with water, and use the finger or a camel's-hair brush. Continue until a complete skeleton about  $\frac{3}{4}$  in. square and of a dirty white color is obtained.

Next bleach the preparation in 1 qt. of water to which have been added a tablespoonful of chloride of lime and a few drops of acetic acid. Place the leaf piece in this for a few minutes, until bleached white. Remove the leaf, blot it gently, and cut out the prepared portion with fine scissors. Transfer this piece to a slide or small stender and stain for 15 min. in cosin, fast green, Lyons blue, or Congo red. Destain as may be required, dehydrate, clear, and mount in balsam.

Best results are secured with leaves collected during dry weather in midsummer, using fully matured specimens.

**Histologic Whole Mounts.**—Certain cells, characteristic of specific tissues of higher animals, can be dissociated from one another and stained to make whole mounts that will reveal shapes and sizes of these individual building blocks. Epithelium, muscle, and nerve cells may be treated in this fashion; blood cells need no such processing and are considered as smears in Chapter 8.

The surface cells of a thick epithelium can be obtained most easily from your own mouth. With a clean fingernail, gently scrape the inside of your cheek and wipe the scrapings onto a clean slide. Add a drop of normal saline and a cover glass for examination in the fresh condition. For

a permanent mount, (1) to the scrapings on the slide add a drop of a mixture of equal parts of 0.5% Congo red in water and of glycerin. Stir with a toothpick to mix the cells with the stain. Cover and seal. Or (2) allow the scrapings to dry; invert the slide over a bottle of pure formalin, fixing the cells in the vapor from this powerful reagent, 1 min., then plunging the slide into 100A 1 min. Stain with hematoxylin and eosin and mount in balsam.

A very fine source for animal epithelial cells to show a surface view is a piece of the skin shed by an amphibian. After frogs or salamanders have been kept for a few days in an aquarium, small patches of thin and almost transparent epidermis will be noted floating in the water. Fix for 6 hr. in formol-acetic-alcohol, rinse, stain in hematoxylin, and mount in balsam, spreading out flat without wrinkles.

Ciliated epithelial cells in the living condition are fascinating objects to watch under high power. In man they occur in various places, lining the windpipe for example, but are not accessible by ordinary means. In the frog, however, these cells extend up into the mouth, from which they may be scraped by gentle manipulations with a fine scalpel blade, held vertically to the surface, without cutting or otherwise injuring the specimen. Mount such scrapings in a drop of normal saline on a slide, stir with a toothpick to distribute the cells over the field, cover, and examine under low power. When one or more cells with actively beating cilia are located, focus on them under high power, cutting down the light. A permanent slide may be made by either of the methods just given for human cheek cells.

Individual cells from such epithelial sheets as the lining of the digestive tract, trachea, or urinary bladder may be procured by one of the several maceration methods, Müller's fluid dissociator being one of the best in the case of epithelia. Place a small piece of the tissue, such as the lining of frog intestine, in this fluid in a small stender for 6 hr., 1 to 3 days for thick epithelia such as skin; then remove to a slide and scrape gently with a scalpel to remove cells

singly and in small clumps. Tease and stir with needles to isolate further and to distribute over the field. When satisfactory, add a cover glass. Now apply a stain, such as Congo red or alum cochineal, with a pipette, adding a drop exactly at the edge of the cover on one side; at the same time or immediately thereafter, draw out some of the fluid with a torn fragment of filter paper applied to the opposite side. This will remove the dissociator and suck in the stain. Drum on the cover glass with a needle in a holder, an action designed to break up clumps and distribute the cells. When the stain has reached all parts of the preparation, cover the slide with an inverted flat stender or other glass vessel which will act as a bell jar; that is, to enclose the preparation so that, as evaporation occurs from under the cover glass, the air within the chamber will quickly become saturated, preventing further evaporation and thus keeping the slide from drying out while the stain is working. If the space enclosed by the vessel is large, it will be well to add a small container of water, alongside the slide, to aid in saturating the contained air; the stain under the cover must not be allowed to dry out. We shall hereafter refer to such a setup as a *humidity chamber*.

After 2 to 4 hr., remove the inverted vessel and proceed with the slide, keeping the cover glass in place. For a glycerin mount, replace the stain with glycerin by the same method used in adding the stain, then seal the mount on the turntable. If this method is selected, it may be as well to spin a very shallow cell for the slide in the beginning. A more permanent slide may be made in balsam but is somewhat tedious, since each step in dehydration, clearing, and mounting must be accomplished by adding the new reagent with a pipette at one side and drawing out the old reagent with filter paper at the opposite side.

We prefer an alternative method, performed in shell vials. It requires a bit more material but the manipulations are far easier. Place the piece of tissue in Müller's fluid in a shell vial, from one-half to three-fourths full, and, during the 6 hr.

allowed for maceration, shake the vial occasionally and vigorously to assist in separating the cells. Then pour the contents of the vial into a flat stender and tear up any remaining chunks of the material with needles. Examine under a lens to see if the dissociation is sufficient; when complete, pour the liquid and contained cells back into a vial. Allow the cells to settle, then decant the fluid, and replace with the stain. Shake occasionally. For every further change of reagents, allow the cells to settle, decant the old fluid, and add the new, finally getting the material into thin balsam. A drop of this is placed on a slide and covered, and should show a sufficient number of well-stained, isolated cells.

Visceral or smooth muscle fibers are elongate, spindle-shaped cells occurring in tubular organs of the body, and best obtained from the wall—not the lining—of the frog intestine. MacCallum's macerating fluid is better than Müller's in this case, and should act for 2 days, with occasional shaking. The nitric acid in this reagent softens connective tissue binding muscle cells together, and makes their complete separation possible, at the same time fixing the tissue. Tease the fibers apart on a slide, preferably with some optical aid, and shred only along the long axis of the fibers; separate them, do not tear them to pieces. When they are sufficiently isolated, replace a quantity of the material in a vial and carry through to a balsam mount by the vial method, staining in hematoxylin.

Skeletal, voluntary, or striated muscle, as this type is variously termed, has large fibers readily seen without magnification. A bit of leg muscle from the frog is a good source. Heart muscle is a special class and a small fragment from the frog heart will provide many fibers. Process both in the manner given for visceral muscle. Mount some of the skeletal fibers unstained, and stain others in Delafield's hematoxylin, iron hematoxylin, or alum cochineal.

For nerve cells, secure small bits of brain and spinal cord (ventral horn of gray matter) from a frog and place in

formalin dissociator for 12 hr., with occasional shaking. Remove the tissue to a slide and add a drop of 0.5% Congo red dissolved in formalin dissociator (instead of in water). Crush the tissue with a scalpel and needles, grinding in rotary fashion; then gather it into the center of the slide, drain off excess fluid, add a drop of glycerin containing 0.5% Congo red, stir to distribute material over the field, cover, and seal. Or use the vial method for a balsam mount, staining with borax carmine, Congo red, fast green, or safranin.

### APPARATUS AND TECHNIQUE

We have met with some of the terms applying to stains and the staining process in Chapter 4, but the topic was not completed there. Stains may be classified according to various schemes:

1. *a.* General stains, as carmine, which colors all parts of the object.  
*b.* Specific stains, affecting only certain parts, as Sudan III is specific for fat.
2. *a.* Basic as to chemical composition, primarily staining the nuclei of tissues, as hematoxylin.  
*b.* Acid stains, as are most of the anilin dyes, such as eosin, coloring cytoplasm.
3. *a.* Nuclear stains, same as 2 *a.*  
*b.* Cytoplasmic stains, same as 2 *b.*
4. *a.* Lethal stains kill cells or microorganisms, as explained in Chapter 2. Methyl green is an example.  
*b.* Vital or intra-vitam dyes are not lethal; example, neutral red.
5. *a.* Aqueous stains are made up as solutions in water.  
*b.* Alcoholic stains are dissolved in alcohol. Eosin may be prepared in either form; acid fuchsin is used in the aqueous form, Lyons blue in the alcoholic.

Staining methods have already been considered, including the use of such terms as "staining in bulk" or "in toto staining"; single, double, or triple staining of sections; counterstaining; and both progressive and regressive staining.

Destaining is essential with the most widely used regres-



sive method; hydrochloric acid is almost universally employed for this purpose, acetic acid being second choice. A few drops of the concentrated acid are added to the wash to make a 0.1 to 1% solution, according to preferences of different technicians; hence the time allowed for the destain to act will also vary, with its strength, but is generally measured in seconds as the acid works very rapidly. Some like to have their destain in water, for example, 100 cc. of water, 5 drops of HCl; others make their mixtures in 35A, 50A, or 70A, and even in 95A. The writer prefers 70% acid alcohol, though choice of the stage in technique when the destain is applied generally makes very little difference. Check the action with the same reagent used as a solvent for the acid; *i.e.*, if destaining with 70 acid alcohol, wash out the acid with fresh 70A. As has already been remarked, it is safer to have this washing alcohol slightly alkaline to counteract any remaining traces of acid.

**Buying, Making, and Keeping Stains, Fixers, and Other Reagents.**—As will be noted in Chapter 17, which deals with formulas and uses of all of the reagents mentioned throughout this volume, some stains are extremely simple to prepare, others more difficult. Where necessary or preferred, any of these may be purchased ready-made in solutions, for immediate use, but are naturally more expensive when thus acquired as you are paying someone else to do the preparation work for you. Not only is it more economical to make your own; the real microscopist will wish to learn to do so for the experience and fun of it. If you buy your own stains ready for use you might as well, by extension of the same principle, buy finished slides and save yourself all of the work. This would, however, involve much greater expense and you would miss all of the fun and learn nothing about microtechnique; it is hoped that you will make up your own stains and learn the whole game from start to finish. These remarks apply equally well to fixing solutions, dissociators, and other mixtures.

The larger apparatus supply houses sell stains in two forms: the dry powder, in 10-g. vials, and the made-up solutions, ready for use. Many of the solutions do not keep well over a long period; the powders last indefinitely. Moreover, so small a quantity of the powder is required to make a copious amount of the solution, that one 10-g. vial of a dry stain, the usual cost of which is 75 cts., should last the average amateur for many years or even a lifetime. If you were to buy no more than one vial of dry stain a month, you would soon acquire an entirely adequate assortment and then have little further expense in the staining department. We suggest that you begin with the most costly and incidentally the most useful, hematoxylin, and add, as needs and finances dictate; eosin Y, water and alcohol soluble, safranin, fast green, powdered cochineal, carmine, one of the violets (gentian, crystal, methyl), Congo red, and Lyons blue. With these as a standard collection, secure such special stains as Wright's stain for blood, iron hematoxylin, the fuchsin, and others as the need for their use in this manual arises.

Of especial interest to amateurs working alone and hence requiring minimum amounts of dry stains from which to make up solutions are the kits of assorted powders recently put on the market by the Hartman-Leddon Company of Philadelphia, manufacturers of Harleco Reagents. These particular dyes are designated as Parstains and are packed in eleven standard assortment units (Fig. 87), titled as follows: Common Dyes, Xanthene Dyes, Basic Dyes, Acid Dyes, Bacterial Stains, Vital Stains, Blood Stains, Fat Stains, Nuclear Stains, Cytoplasmic Stains, and General Stains for Plant Histology. For an average cost of less than \$2 one may obtain a set of 10 capsules, each containing the correct amount of dry stain from which to make up 50 cc. of solution. No weighing is necessary; simply empty the contents of a capsule into 50 cc. of the solvent, water or alcohol, as the case may be.

Most stains can be used repeatedly. Use them until they

“wear out” as evidenced by poor results. Hence it is good practice to have three sets of bottles for your stains: the original vial containing the dry powder; the bottle of unused solution; and a bottle containing used solution and marked



FIG. 87.—One of the Harleco Parstains standard-assortment units. (*Hartman-Leddon Company.*)

as such, *e.g.*, “Used Lyons blue.” Keep these three sets in separate places or on separate shelves, arranging each set alphabetically. Never pour used stain back into the unused bottle.

Alcohols and clearers may be used over and over also, but not fixers, which should be discarded after each usage. Maintain separate bottles for the used alcohols and clearers. It is customary in laboratories to arrange a row of bottles of fixing solutions, alphabetically, some of which are ready for use just as they are; others need the last-minute addition of some chemical that cannot be allowed to stand with the others for any length of time since it would bring about a deteriorating or changing reaction. Acetic acid in fixing mixtures is one such. The balance of the formula, excluding the acetic acid, is made up and termed a “stock solution.” It is good practice to give the formula on the label, as one does not then have to refer to a manual to refill; if this is done, another label should state what should be added to complete the formula.

As an illustration, let us take Zenker's fluid, one of the most efficient and widely used of all fixers in animal histology. The quantity to be made up at one time for stock will depend on how much of it the individual technician will probably use in the course of a year. Professional and college laboratories would of course consume a great deal more than the independent amateur. In any given usage, the rule is to take never less than ten or more than a hundred times the bulk of the object to be fixed. Thus, if the object measures roughly 1 cc., you should use somewhere between 10 and 100 cc. of the solution to fix it properly. Most experts advocate using copious amounts of fixer; always use too much rather than too little. In practice it is not customary to measure the amount used since the exact amount is unimportant; simply use an adequate amount.

Let us assume that your own needs require making up Zenker's stock in 500-cc. quantity at one operation, saving time in having to prepare it too frequently, and that you have a bottle of suitable size for this amount. Put a label on the bottle reading as follows:

ZENKER'S FLUID STOCK

Mercuric chloride.....	25.0 g.
Potassium bichromate.....	12.5 g.
Sodium sulphate.....	5.0 g.
Water.....	500 cc.

Below this is pasted a second label, reading,

TO USE

Add 5 cc. glacial acetic acid to each 100 cc. stock.

Used alcohols, after they have been worn out in the routine techniques, and of 70% strength and up, may be poured together in one container, filtered through cotton or

cheesecloth, several folds, and labeled as "Used Strong Alcohols," to be used in cleaning glassware, instruments, and brushes that have been in shellac, for wiping off the table top, and for other miscellaneous purposes.

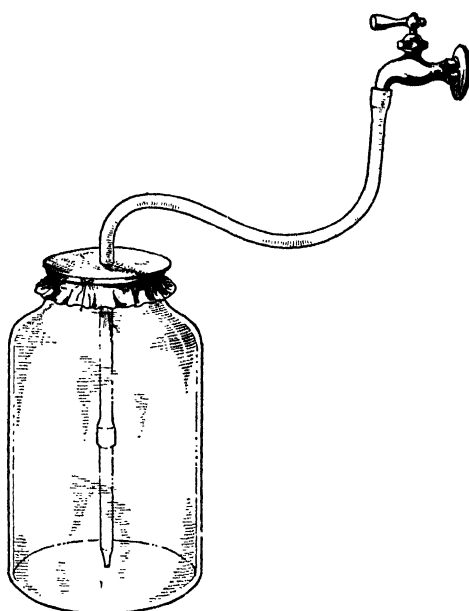


FIG. 88.—Setup for washing tissues in running water.

**Washing in Running Water.**—A simple method for small quantities of material is to rig up a washing bottle. A fairly large wide-mouthed bottle is closed with a two-hole rubber stopper. A long glass tube reaches almost to the bottom of the bottle and delivers a very gentle stream of water from the tap; it should be so slow as to cause no disturbance to the tissue or animal—just a bit more than a drop-by-drop delivery. The runoff is cared for by the shorter tube, the hose from which leads to the sink. Instead of a rubber stopper, the neck of the bottle may be covered with gauze held by a rubber band around the bottle neck, and the tube from the tap merely punched through this (Fig. 88). No second tube is necessary, the runoff flowing out through the

gauze, which prevents the specimen from being washed out of the bottle.

A more elaborate apparatus, suitable for school laboratories or for use where a number of different preparations are to be washed at once, consists of an outer tray or tank, preferably of copper, within which rests a compartment tray, also of copper, with divisions or boxes made of perforated copper sheeting or gauze. Delivery of water is through a hose from the tap, attached to a nozzle soldered into the outer tray, which rests in or drains into a sink. Any sheet-metal worker can make this outfit at no great cost.

**Narcotizing Methods.**—In order not to have to attend continually to the duty of adding more narcotic to the water in which specimens are being killed, it has been suggested that the epsom salts or other reagent be placed in a small cheesecloth or muslin bag, which is then suspended above the vessel of water so that the bottom of the bag just barely touches the water. The salt will then diffuse slowly out of the bag into the water, gradually increasing the dosage without further attention.

Other recommended methods include a capillary siphon or one using a piece of string or fine wick. A beaker of the salt in strong solution is elevated above the vessel of water containing the specimens, and either a siphon of glass tubing of capillary fineness, or a wick or string is led from the beaker to the vessel, delivering a constant but small amount of the salt solution so that the strength of the salt in the main vessel gradually increases.

For precise methods, as with research materials, some technicians prefer to change all their reagents in the regular technique by one of these steady and slow procedures. Thus, instead of going through a graded series of alcohols during dehydration, with abrupt changes between 35, 50, 70, 82, and 95 percentages of alcohol, they start the material in water in the lower vessel and place 95A in the upper beaker, with one of the several forms of siphon adjusted

so that 12 to 24 hr. is required to exhaust the supply of alcohol. The lower vessel may have a similar siphon, or other form of runoff into the sink, resulting in a complete change from water to 95A by the end of the period. Such dehydration is very gradual and perfect but not necessary in all ordinary work.

## CHAPTER 8

### SMEAR PREPARATIONS

*In This Chapter:* permanent stained slides of protozoa and algae; the iron-hematoxylin and venetian-turpentine techniques; pure cultures of protozoa; smears of blood, fibrin, hemin crystals, spermatozoa, and pollen grains.

**A**MONG the most important as well as difficult of slides covered in this chapter are the stained and permanent mounts of protozoa, with which we shall begin. Such a slide is never so interesting as a water mount of the living animals; there is always a certain amount of distortion and an unnatural appearance to even the best of them, but they also have some advantages, permanence being one. Of greater importance are the staining and bringing out to clear visibility of several structures seen but faintly if at all in the living cells, as nuclei, flagella, and various minute bodies (Fig. 89).

**A Mixed Culture Smear.**—There are two general methods for handling materials: as films or smears on cover glasses, and as sediments in vials (*sedimentation method*), just as has been explained in Chapter 7 for epithelial and other cells under the heading of Histologic Whole Mounts. In fact the preparations under that heading could just as well have been included here under smears.

Zebrowski's rapid method is perhaps best for the beginner and gives good results for general purposes. He makes a film of Mayer's albumen fixative on a cover glass and on this places one or two drops from the selected culture of protozoans, spreading the water over the cover until in a thin film by using a toothpick, broomstraw, or cigarette paper, and draining off excess water by tilting. While the



cover is still moist, he passes it once through a flame, film side up, killing the organisms by heat, and then places the cover face down on the surface of a small stender of the fixer—hot, but not boiling, saturated aqueous solution of

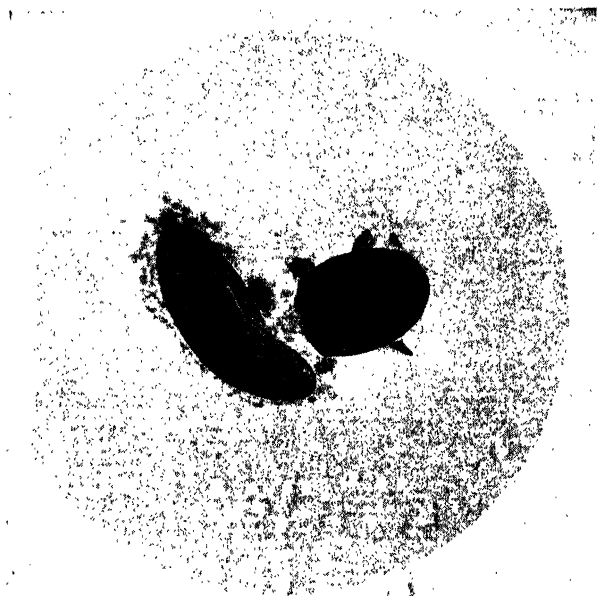


FIG. 89.—The drama of life and death in the microcosm. Didinium, turtle-shaped paramecium tiger, is here caught in the act of devouring Paramecium, the slipper animal.

mercuric chloride. Handling with fine curved forceps, he turns the cover face up before it sinks and leaves it in the fixer 3 min. Wash well in distilled water, then place the cover face up on a slide and pipette on a strong aqueous solution of iodine for 3 min. Again rinse in water, then transfer to a 2% aqueous sodium thiosulphate solution for another 3 min. Rinse in water and stain in borax carmine for 5 to 10 min., rinse again, then dehydrate rapidly, about 15 sec. each in the ascending alcohols. Add enough fast green to the 70% alcohol to give it a distinctly green color,

and go from 95A into carbolxylene, pure xylene, and mount in balsam. Nuclei are stained red, cytoplasm green.

**Schaudinn-Iron-hematoxylin Technique.**—This famous method may be employed with all protozoan smears and many algae, but is considered especially effective in the case of fecal smears, such as are used to determine the presence of intestinal protozoa. An example will carry us into the field of medical parasitology.

A very small bit of fresh human or animal feces is taken up on the blunt end of a toothpick and rubbed on a slide in a drop of normal saline to make a thin film. Prepare the film on a cover glass if preferred. If much of this work is to be done, the operator should invest in a wire loop, as described in Chapter 9. Reagents are pipetted onto the slide, if this method is employed; if cover glasses are used, handle as was done in the preceding method. We shall assume the smear to be made on a cover glass in the following explanations.

Drop the cover, film side down, on warm Schaudinn's fluid (at 40 to 45° C.) for 15 to 30 min. A No. 1 cover will float on the surface of this and subsequent reagents and expose the protozoans to the various actions. If the smear does not adhere properly to the cover, treat the glass first with albumen fixative. Without washing, the film is next treated with 35A and 50A, 5 min. each, then with 70% iodinated alcohol for 20 min., 82A for 3 min., and 95A for 5 min.

When ready to stain, the cover is hydrated by passing it down through the alcohol series, 2 to 3 min. in each, into water for a few minutes, then into solution I, iron hematoxylin, for some 6 hr., then distilled water for 10 min., and solution II of the stain for another 6 hr. Solution I in this combination is ferric alum (ammonioferric sulphate) and acts here as a *mordant*, that is, it does not stain but prepares the material to take a stain. Solution II is the stain, ordinary

hematoxylin. The method is regressive and purposely overstains the objects which must next be *differentiated* (destained) by a second immersion in solution I. From the hematoxylin, pass the cover glasses to water, 5 min., then to a separate container with fresh solution I (not the jar used the first time) for 10 to 30 min., the length of time varying so widely with different subjects that no set rule can be given. The operator must determine for himself the proper time with each type of preparation; hence it is customary to prepare several films in advance, using the first one or two as trial horses which may or may not turn out satisfactorily, then applying the experience so gained to the staining of extra films and ending up with one or more really fine slides.

The usual procedure is to remove a given cover glass from the destain at intervals, dip for a moment in water, then mount on a slide face up under the microscope for a quick inspection. Be sure to keep it wet with water at all times. When, by such examinations, the sharpness of the staining is judged to be correct, check the destaining action by washing the cover in tap water, 2 hr., preferably in running water. Dehydrate, clear, and mount in balsam. No counterstain is used with protozoan slides in this technique.

Iron hematoxylin, often abbreviated to "iron h," is a somewhat advanced and tricky technique for the beginner to attempt and is more easily mastered with sectioned material than with smears. Once conquered, however, it seems no more difficult than many another task, and yields results for certain purposes superior to any other method. It has become most celebrated as a stain for chromosomes in the study of cytology, of great significance in embryology, genetics, and other fields of modern research, but is also one of the best stains for protozoa, algae, blood, striated muscle, and other subjects.

In order that the amateur who is working alone and has no means of supervision may get an idea of what con-

stitutes a properly differentiated iron-hematoxylin slide, it would be well for him to purchase one or more from a reliable biological supply house, or inspect such slides in the collection of a high school or college biology department. The following are recommended: one slide of striated muscle fibers; one of mitosis (cell division) in a plant root tip or salamander or grasshopper testis; and one fecal smear. In the last case, take advantage of the opportunity to purchase a species difficult to obtain in any other way, the celebrated *Endamoeba histolytica*, a parasitic relative of amoeba causing amoebic dysentery in man. A good slide of protozoans should show the nuclei and certain other cellular bodies from jet black to a dark blue black; other objects in the field are unstained.

Sometimes destaining in the mordant imparts a dirty gray to the cytoplasm of cells; for this reason many workers prefer to differentiate in a saturated aqueous solution of picric acid. Among other fixers widely used for protozoan smears are Bouin's, Carnoy's, Gilson's, and Zenker's; for staining, various technicians use Delafield's hematoxylin, Mayer's hemalum, safranin, acid fuchsin, or methyl green, with or without contrasting counterstains.

In the sedimentation method, a pipetteful of the desired protozoans is shot quickly into a shell vial half filled with the fixer, and all subsequent treatments of fixing, washing, staining, destaining, dehydrating, and so on to and including mixture with a thin balsam are carried on in the same vial by the principle explained in preparing epithelial cells in Chapter 7.

**Pure Cultures of Protozoa.**—The general procedure used by those engaged in working with protozoa is first to secure *natural cultures* from ponds and other sources, which are inevitably *mixed cultures* as to species present; then to derive *pure cultures* from these, and finally to make *sub-cultures* from the pure cultures from time to time as may be necessary to maintain them in a healthy condition. All

glassware and instruments used in this work should be sterilized, according to directions in Chapter 9 for similar routines with bacteria. Detailed or advanced work requires a binocular microscope.

Pond water, together with algae and other aquatic plants and some of the bottom mud, is collected and small amounts placed in specimen dishes, finger bowls, flat stenders, or petri dishes, covered with a glass plate, and put in diffused daylight but not in direct sunlight. Have available a separate and labeled pipette for each dish, to avoid contaminating one with the contents of another. Make microscope examinations from time to time, noting what forms are present on the bottom, the upper surface, the sides, and the central fluid. Whenever a particular and wanted specimen is found, pure cultures are in order.

Distilled water is placed in a specimen dish and several strands of boiled hay added, on which bacteria will develop and serve as food. After two days, inoculate with the desired protozoan. LaRue recommends a mouth pipette, made by inserting a glass tube with a finely drawn tip in one end of a 12 to 15-in. length of rubber tubing and with a short section of glass tubing as a mouthpiece. Using a binocular microscope or the low power of your compound instrument as the nearest practical substitute, bring the point of the pipette close to the protozoan and suck in at the mouthpiece. With a bit of practice, single specimens can be captured in this manner and allowed to flow out onto a slide. Inspection will then show whether other and unwanted organisms are present, and the process repeated until the single desired individual is isolated and placed in the prepared culture dish. Detailed directions on the proper care and feeding of particular species are to be found in the reference work, *Culture Methods for Invertebrate Animals*.

Even the best of pure cultures tend to go stale and die out; if one wishes to maintain the given organism for a long period, some of the flourishing group that develops from the

single original cell should be transferred from time to time to fresh culture dishes. This is known as "subculturing."

**Smear Mount of Algae.**—Many of the unicellular algae may be handled much as was done with the protozoa. As an example, and incidentally introducing another technique combination, let us prepare a smear of pleurococcus (protococcus) by fixing with chrom-acetic acid, staining with phloxine and counterstaining with anilin blue.

This species occurs in moss-like or felt-like growths on trees, especially at their bases, and on boards, fences, stones, and other moist situations. It prefers a northern exposure to such an extent that it has been used frequently as a compass by those lost in deep northern woods, where the green scummy growth of pleurococcus abounds. Collect it by scraping off a bit of the bark or wood containing the algae and, arrived at your workroom, scrape some of the cells into water. The material may be handled by the sedimentation method throughout or affixed at the start to a slide coated lightly with albumen fixative.

In either case, fix in chrom-acetic 24 hr., wash in running water 24 hr., dehydrate to 95A, and stain in phloxine 24 hr. Then rinse in 95A and counterstain with anilin blue for 2 to 10 min., sometimes longer. It is best to determine this time by a trial with a small part of the material before subjecting the whole lot to this stain. Rinse in 95A, then destain with 95 acid alcohol, which will extract the red but intensify the blue; hence a bit of experimentation is needed to get just the right results. Excess blue can be decolorized with straight 95A. If the red becomes too dull, treat with a wash of 95A to which a bit of sodium carbonate has been added to render it slightly alkaline. Pass through 100A for a few seconds, clear in xylene, and mount in balsam.

**The Venetian Turpentine Method.**—Filamentous algae, such as the well-known pond scum, spirogyra, give considerable trouble by routine methods during dehydration,

and especially while clearing and mounting, being prone to collapse when placed directly into balsam. This mountant can be used if, from 100A, the specimens are passed through about 10 different mixtures of 100A and xylene, gradually increasing the strength of the clearer, such as beginning with 9 parts 100A to 1 of xylene, then 8 to 2, 7 to 3, and so on to pure xylene, about 5 min. each. Use three different changes of xylene, then place in an extremely dilute xylene balsam or, even better, damar balsam, one-fifth or less of the usual concentration, and allow this to thicken for some 2 days in the evaporator, then mount. Since the transfer into balsam is the most critical stage, some failures are to be expected. One of the siphoning or wick methods of effecting a gradual replacement of one reagent by another can be used here for getting the algae into xylene, if preferred.

Venetian turpentine is the resin of the European larch (*Larix europaea*) and is similar to Canada balsam in appearance and some of its reactions, but is miscible with 100A; hence material can go directly from this alcohol into the mountant without the use of a clearer. The technique is tricky and needs some practice gained by experience, during which some failures are to be expected. As given by Chamberlain, the steps are as follows:

Fix some filaments of spirogyra in chrom-acetic for 24 hr., using a volume of fixer at least fifty times that of the specimens. Wash in running water for 24 hr.; solution I, iron hematoxylin, 4 hr.; wash in running water, 20 min.; solution II, iron hematoxylin, overnight or 24 hr.; wash in running water, 20 min.; solution I, iron hematoxylin, to differentiate until the stain is satisfactory, as determined by frequent inspection under the microscope. Wash in running water, 2 hr.

Transfer to a petri dish of 10% glycerin in distilled water and place uncovered in the evaporator until the glycerin is concentrated by evaporation of the water. This may take 3 or 4 days, but may be hastened by gentle warming, not

over 35° C. In any case this concentration should not take less than 2 days, or the material will suffer. Wash out the glycerin with 95A, changed several times, over a period of 10 to 20 min., and save this alcohol for cleaning dishes that have held venetian turpentine. Place the specimens in 100A for 10 min.

As Chamberlain emphasizes, most failures are now ready to occur, this being a critical point in the technique. Prepare a desiccator, also known as an "exsiccator," which is the opposite of a moist chamber and is a piece of apparatus designed to dry objects or to process them in an atmosphere of absolute dryness.

The desiccating agent may be soda lime—a mixture of sodium hydroxide with quick lime—or fused calcium chloride, or sodium hydroxide, all of which will absorb moisture from air with great intensity. One may purchase a suitable piece of glassware (Fig. 90) or make his own desiccator by simply placing a saucer or dish of the drying agent in the bottom of any tight-fitting receptacle, such as a vacuum coffee can, or by covering the dish with a bell jar or inverted finger bowl.

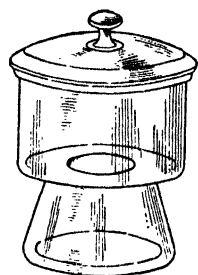


FIG. 90.—Desiccator.

With the desiccator in readiness, pour the 100A off the material and place the vessel containing the spirogyra in the desiccator alongside the saucer of drier; then pour 10% venetian turpentine on the algae and *immediately* cover the whole. As this turpentine is 90% absolute alcohol and 10% turpentine and is extremely sensitive to moisture, it will pick it out of the air and turn milky, hence the reason for performing this step in perfectly dry air. Do not allow any of the drying agent to get into the turpentine dish. Concentration now occurs, the material being left not less than 2 days and continuing until the turpentine has the consistency of pure glycerin, when it may be exposed to air safely. If it becomes too thick, thin it with more 10% turpentine or a few drops of 100A. Mount in a drop or two



of the venetian turpentine, which hardens like balsam and is permanent. Tapping on the cover glass with a needle will often separate filaments that have come together.

**Permanent Blood Slides.**—The blood of any animal may be used, human blood being obtained by the methods given in Chapter 2. A collection of slides showing bloods of each of the five main classes of vertebrates is most interesting and instructive. Fixation is best accomplished by rapid exposure of very thin films to air, by the following method, which must be practiced until thoroughly mastered.

Secure a small drop of fresh blood on a clean cover glass and instantly cap it with a second cover glass, and immediately slip the two covers apart sidewise, without either pressing or lifting. Slide one off the other so as to leave a uniformly thin film on each and wave them in the air, one in each hand, until completely dry. Speed is the requisite here, to fix the corpuscles in their natural condition, without crushing or distortion, and so quickly that post-mortem changes and coagulation do not have time to take place. If you like, practice film-making with red ink until facility and speed are gained. Some prefer to hold the two covers with the fingers, grasping each by the edges only; others use a cover glass forceps for one of the films, the fingers for the other; still others employ two forceps. The fingers are best, however, time being wasted in manipulating the forceps.

When the films have dried, place one of them flat on the table or in the grasp of a cover glass forceps, film side up, and reserve the other film for a second preparation later. Cover the blood smear with a measured quantity (*i.e.*, one or two drops) of Wright's stain, timing the duration accurately to 1 min. Then, without removing the stain, add an equal quantity of distilled water; that is, if you used two drops of stain, add two drops of water. Allow this to remain two additional minutes, then wash and differentiate in distilled water until the film as a whole has a salmon-red

color. The water acts as a destain and its action must be checked or all color will be removed. Under the microscope, red corpuscles should be salmon or pinkish; the nuclei of various leucocytes show purple or blue. Consult a textbook

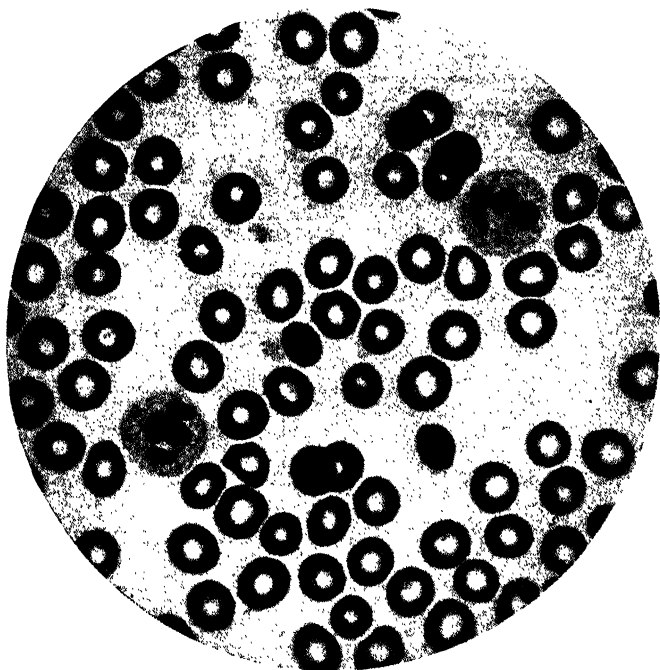


FIG. 91.—Human blood, Wright's stain, 800 X. Two neutrophils, a form of leucocyte or white blood corpuscle, are seen surrounded by many erythrocytes, the red blood corpuscles.

of histology that has a colored plate showing the appearance of various corpuscles as correctly stained with Wright's and practice until a perfect slide is obtained. Longer staining or shorter washing yields bluer slides; shorter staining or longer washing gives redder results. Figure 91 is a high power photomicrograph of stained human blood.

When sufficiently differentiated, blot the film gently with a piece of filter paper; when completely dry, mount in balsam which, for this purpose, must be neutral. To be sure that the balsam is not acid, some technicians keep a few

small pieces of marble in the bottom of their balsam bottle permanently. A still better mountant for blood films is euparal or diaphane, which intensify and preserve the colors for years, giving a more brilliant slide.

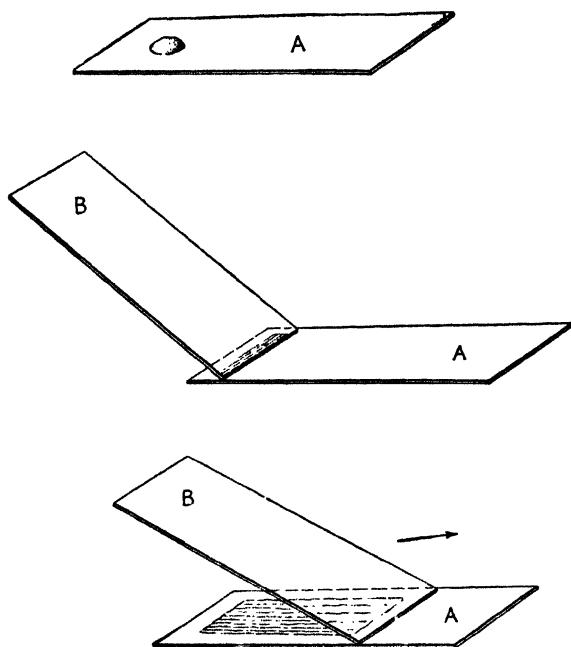


FIG. 92.—Steps in making a blood smear on a slide.

Some technicians, particularly in hospitals, prefer to make smears on a slide instead of on a cover glass, thus securing a greater area, with which a large rectangular cover glass is employed, or the dried film is left permanently uncovered for use with an oil immersion lens. To follow this procedure, place a large drop of blood at one end of a slide which lies flat on the work table and which we shall designate as slide *A* (Fig. 92). Holding another slide (slide *B*) in the right hand, inclined at a bit less than a 45-deg. angle to slide *A*, rapidly make contact with the drop and push slide *B* along slide *A*, away from the drop. The blood spreads out into a line where the two slides make con-

tact and is pulled into a film by following the retreating slide through capillarity. This method, too, must be practiced until it can be performed with ease and rapidity, so that the film will be made and dried before any changes can set in. One advantage is that no chance of crushing the corpuscles occurs. Such films are dried and stained as in the cover glass film method. Decreasing the angle at which the two slides meet makes a thinner film, and vice versa.

Wright's stain is excellent for malarial parasites and will also stain trypanosomes, but not microfilariae. For the latter use iron hematoxylin or the eosin-methylen blue combination. There are many other fixers and stains for blood, to be found in larger manuals, but they are generally more difficult to prepare and use. For work with blood parasites, leave the film uncovered. After examination with an oil immersion lens, cautiously wipe the excess oil from the film surface with a bit of lens paper, taking care not to wipe off the film itself. The small amount of oil remaining does no harm. Uncovered films retain their stain much longer than covered ones.

**Blood Fibrin.**—Blood is a most complex fluid, containing many parts in addition to the corpuscles. Among interesting slide preparations that can be made is one to show the fibrin, which forms upon exposure to air and thus assists in clotting. Instead of speed, we need just the reverse here, to allow the fibrin to form. A drop of blood is drawn and mounted on a slide under a cover glass as if for examination of fresh blood, and the slide is placed in a humidity chamber to stand for half an hour. This chamber, the opposite of a desiccator, was described in Chapter 7, in the mounting of epithelial cells.

In this case, where a slide is used, make a humidity chamber by inverting a finger bowl or similar item of glassware over a china plate on which is a thin sheet of water. Put two short sections of glass rod in the plate and

place the slide across these rods so that it is elevated above the water level; any other convenient supports may be used. After 30 min., place a few drops of water around the cover glass of the blood slide; as soon as the cover glass is suffi-



FIG. 93.—Hemin crystals prepared from human blood and photomicrographed by dark-field illumination.

ciently loosened, remove it carefully with fine forceps. Now irrigate the film fully but cautiously with distilled water, without washing the preparation off the slide, drain, and stain for 3 min. with a 1% aqueous methyl violet; drain, rinse with water, allow to dry, and mount in balsam.

**Hemin Crystals.**—Here is another blood slide that all microscopists will wish to make. These bodies are very small, rhombic, brown plates which occur singly, in crosses, or in star-shaped groups, and constitute a positive indi-

cator of the presence of blood (Fig. 93). Hence they are of great importance in criminological cases where the nature of a suspected stain is under investigation, to determine whether or not it is blood. The method may be used on old and dried stains, on bits of clothing, or on scrapings from stains on furniture or floors just as effectively as for fresh blood. No other known substance will yield such crystals.

*Sodium Reaction:* Place a drop of blood or any material containing a dried bloodstain, such as shreds of cloth, on a slide and sprinkle on a few grains of sodium chloride (table salt). If dried material is used, add a drop of water or normal saline. In either case, heat over an alcohol lamp slowly until dry. Put on a cover glass and, with a pipette, add at the edge of the cover enough glacial acetic acid to float the cover. Heat again, boiling the acid slowly and gently until it has all boiled away; then remove the cover and examine the slide under the microscope. If hemin crystals are present, add balsam and a cover glass to make a permanent mount. Not every slide will be successful.

*Potassium Reaction:* An alternative method is to add to the blood after it has been dried on the slide two drops of Nippe's solution, made by dissolving 0.1 g. each of KCl, KI, and KBr in 100 cc. glacial acetic acid. Put on a cover glass, heat gently until dry, examine, and mount in balsam with a clean cover if successful.

**Spermatozoa.**—From a freshly killed male animal, *e.g.*, a rat, cut out a portion of the vas deferens or a slice from the testis and smear the cut surface upon a slide or a cover glass. Seminal fluid may be obtained from large species, as the horse, by the use of an artificial vagina, as is frequently practiced in pedigreed stockbreeding. Examine living spermatozoa in a drop of normal saline, using the highest power available and cutting down the light.

One small drop of seminal fluid will make a cover glass or slide film as directed for blood. Either films or smears are dried in the air or by placing on a hot plate; a safer method

is to immerse a cover glass or slide containing the smear in corrosive sublimate for 15 min., washing in water, dehydrating through 35A, 50A, and 70A iodinated, then hydrating back to water, and staining for 1 hr. or longer in safranin. Wash in water and dehydrate rapidly through the alcohols, 30 sec. each, clear, and mount in balsam. Wet films or smears should be handled on slides coated lightly with albumen fixative. Guyer recommends staining fixed sperm smears in cyanin for 10 min., then counterstaining in erythrosin.

**Pollen Grain Smears.**—Dried pollen is shaken upon a slide and xylene added to remove the coating of oil often present. Twist a very small and flat bit of cotton around the tips of a pair of fine forceps, seeker, needle, or toothpick, making what is called a “pledget,” and with it wipe off superfluous xylene together with the extracted oil. This process may have to be repeated several times. Now to a small amount of anilin add sufficient stain to color this oil rather deeply, using either fast green, gentian violet, crystal violet, or other anilin dye as the stain. Put a few drops of such tinted anilin oil on the slide, stirring in the pollen grains. Heat over an alcohol lamp until the oil steams but do not boil. Observe from time to time with a hand lens and keep up the steaming until the pollen seems sufficiently well stained. Allow to cool and wipe off excess oil with another pledget; then treat with xylene two or three times, wiping off after each treatment or drawing off with a bit of cotton or filter paper to get rid of excess oil and dye; finally, while still damp with xylene, add balsam and cover.

## CHAPTER 9

### BACTERIA

*In This Chapter:* observations and slide-making without culturing equipment; manufacture of sterilizers, incubators, and other items for advanced studies; preparing culture media, tubing, incubating, inoculating, plating, streaking; special staining techniques.

**N**O ONE of the many fields embraced by microscopy has commanded so much public attention in recent years as bacteriology, nor has any other witnessed a greater scientific drive. All over the world, wherever modern civilization has penetrated, laboratory technicians keep ceaseless vigil to discover the ways of living—and of dying—of these organisms whose importance is inversely proportional to their size. And the amateur, like his professional brother, finds this study unexcelled in absorbing interest and clamors to know how to undertake the exploration of this invisible world which lies about us everywhere and even penetrates to the remotest crevices of our own bodies.


Since the science has become so advanced that an entire volume would be needed to do the subject justice, no attempt is made here to cover bacteriology at all fully; the reader is referred to any of a number of excellent texts and manuals, widely available in all libraries. Moreover, the beginner is cautioned to make progress slowly and to look ahead and see what he is getting himself in for, rather than to attempt to plunge in blindly. A little knowledge is a dangerous thing and may give the novice an exaggerated opinion of his own powers. We suggest that you first proceed along the lines laid down in this chapter and then, if desirous of going further, to seek instruction and practical experience under the guidance of a qualified bacteriologist. Many summer positions are open to both men



and women of high school and college age and training, in city laboratories and hospitals, where routine duties are performed and advanced work learned under proper surroundings and safeguards.

Bacteria are unicellular plants belonging to the fungi. Each cell is a single plant, just as much so as a rosebush or a pine tree, and reproduction occurs by fission, a splitting in two. There is no centralized nucleus, the chromatin material being scattered through the cell in fine granules. These organisms take the form of rods (bacilli; singular, bacillus), spheres (cocci; coccus), or spirals (spirilla; spirillum), and were originally described as distinct species on a basis of morphology (structure), as is commonly done with other plants and with animals. Thus we have streptococci (spheres in chains), staphylococci (spheres in bunches like grapes), mycobacteria (growing like a fungus), and so on. More recently it has been found that appearance of cells under the microscope cannot always be relied on, that under given conditions certain types can change their shapes from one form to another, and that what were formerly considered two distinct species may be no more than two phases of one. Hence reliance has come more and more to be placed today on culturing methods and the growth behavior of colonies visible to the unaided eye; the color, size, and shape of growth on such diverse media as potato, bouillon, agar, and other nutrient substances giving information that either supplements or replaces microscopic studies.

Another marked shift in emphasis, as bacteriology has progressed from its days of infancy among the sciences, is the recognition that by no means are all species harmful, and that relations of bacteria to the rest of the world, inorganic as well as organic, follow along lines pursued by other organisms. There are species dwelling in soil that are able to capture nitrogen from the atmosphere and thus enrich our farms; others bring about decay and return simple elements to the land. Certain types make possible many useful commercial processes, such as the retting of



flax, the tanning of hides, or the manufacture of cheese. Many bacteria are neutral as regards man. Finally there are those destructive species that spoil human foods through the souring of milk or the decomposition of meat, culminating in the spectacular forms that have found their best living conditions on or within the bodies of higher plants and animals. Here, unless centuries of such foreign invasion have brought about an adjustment on the part of the species thus invaded (the host) to the ravages of the interloper (the parasite), such an adjustment being a natural immunity, there will be either an active damage in the form of destruction of tissues or a passive one in the liberation of poisonous substances (toxins) by the parasite. Such ravages make themselves manifest as symptoms of specific diseases, some of the more notable being, among plants, the blights and wilts of apples, beans, peas, cucumbers, and alfalfa, the rot of cabbage, and the galls of tomato; among animals, the cholera of fowls and hogs, the plagues of cattle and swine, the foul brood in the honeybee, and such dreaded human infections as diphtheria, bubonic plague, Asiatic cholera, syphilis, gonorrhea, tuberculosis, typhoid, leprosy, and tetanus. Bacteria causing diseases are known as "pathogenic" bacteria, or simply "pathogens."

The amateur is cautioned that he must confine his attentions to the non-pathogenic species unless he works under the direction of a professional bacteriologist, or until his knowledge and skill have attained the professional level. Since the harmless types are fully as interesting to work with, there is no excuse for flirting with the undertaker by handling deadly species simply because they are deadly.

One drawback to this work on the part of many of our readers will doubtless be the lack of an oil immersion lens which, with a 10 X eyepiece, will give a magnification above 900 X, necessary for observation of many forms and for detailed study of nearly all. This lens must be used with a substage condenser for proper resolution; hence it involves the more expensive optical equipment which

probably only the minority of our readers will possess. However, there remain certain observations that anyone can make without professional equipment and with any microscope having a magnifying power of 300  $\times$  and up.

**Observations of Bacteria without an Oil Immersion Lens.**—Prepare a clean slide and cover, and on the slide place a drop of saliva from your mouth. With a clean fingernail, gently scrape the inside of your cheek, or with a toothpick remove some of the matter that accumulates between the teeth. Mount either of these substances in the drop of saliva and cover. Cut down the light and observe, watching for minute, long rods, which may occasionally be seen in motion. This is leptothrix, a giant among bacilli, which lives as a commensal in the mouths of nearly all persons and, as far as known, does no harm at all.

Prepare a hay infusion, as directed in Chapter 2; after a day or so, an immense swarm of bacteria will be found feeding on the wisps of grass and bringing about decay. It is these bacteria that serve as food for the many protozoa that also appear in these infusions. Lettuce or many other plant materials will serve. It is essential to cut down the light as much as possible, whereupon vast numbers of the bacteria may be seen chiefly through the commotion they make in the drop of water mounted on your slide. Each cell is barely visible at 300 to 500  $\times$ , but the mass of them should be made out with ease once you have become accustomed to the dim light and the search for anything so very small.

Other good sources of bacteria in nature may be found in the following: place a dead fly in a flat stender or watch glass of pond water and observe a drop of the water after a day or so; allow a small piece of raw meat to decay in a very little water in a dish; collect some water from a sewer, if an outlet can be found, or from a stream that is polluted with sewage; secure a very tiny bit of feces, human or animal, and prepare a smear as was done in Chapter 8;

sprinkle a pinch of dirt from the floor over a small amount of water in a watch glass.

The hanging-drop method is much used in the study of bacteria. Prepare, as directed in Chapter 2, the cover glass sealed on with vaseline. Focus on the edge of the drop with low power, then change to high, using care not to break the cover glass.

When handling any materials such as sewage that might be dangerous, observe the simple precautions of the bacteriological laboratory: always wash the hands thoroughly both before and after making any experiments or observations; during such studies, keep the hands away from the mouth, face, and clothing; boil all glassware and instruments used after completion of studies; if any materials are spilled, clean them up carefully and throw the rag away.

**The Simpler Standard Methods of Mounting and Staining.**—The wire loop, essential in this work (Fig. 94), until recent years could be obtained only in the expensive form of platinum wire. Such an instrument is still popular, but amateurs and others who will use a loop only occasionally can secure one made of the much cheaper nichrome wire. Both before and after each individual use of a loop, it is *flamed* by heating to a red color in the apex of a flame to ensure its sterility. The smears are customarily made on cover glasses, though they may be prepared on the slide if desired.

Clean and flame several slides and covers and have them ready for use before you on the table, one cover held, preferably, in a cover glass forceps. Flame the loop and, without laying it down or touching any other object with it, pick up a loopful of any fluid at hand that contains bacteria and spread it in a thin film on the cover glass held in the forceps. Allow it to dry in the air; then kill and fix by passing the cover, film side up, three times rapidly through the apex of a flame, about  $\frac{1}{2}$  sec. each time. If no loop is

available, make films as directed for blood in Chapter 8. If the bacteria are on solid media, gently skim the surface with the loop and rub up in a drop of distilled water on the cover glass.



FIG. 94.—Inoculating loop.

A rapid staining method is to drop on gentian violet (or methyl violet or crystal violet) for 5 min., rinse by allowing a very gentle stream of water to trickle over the cover from a tap, plunge into 95A for a moment to destain, drain off excess alcohol, blot gently and carefully with a piece of filter paper, flame to complete drying, and mount in balsam.

A somewhat longer and more exact technique is the Gram stain, not only excellent in itself but serving also as a differentiator. Treatment with an iodine solution prepares certain kinds of bacteria so that they retain the gentian violet during decoloring; others give it up. Those species keeping the stain are termed Gram-positive bacteria; those losing the violet are Gram-negative—a useful diagnostic test. Prepare a film and proceed as before with the staining in violet and rinsing in water. Now flood the cover glass with Lugol's iodine solution (also termed Gram's solution) for 2 or 3 min. until the stain is black; then wash with 95A until the stain ceases to come away freely—usually 30 sec. to 1 min. Rinse in water again and counterstain with a 1% aqueous solution of safranin or basic fuchsin for 10 sec. Wash off excess counterstain in water, dry by blotting, and mount in balsam; or rinse in 100A, clear in xylene, and mount in balsam.

Some experimentation with this technique is necessary to get the intensity of the violet color just right; most workers prefer to examine the cover glass under the microscope, mounting in a thin film of water for temporary observation. If the violet is too light, repeat or discard and

stain the next cover longer or differentiate for a shorter time in the 95A; if too dark, bleach further with the alcohol.

Many technicians prefer methylen blue as the primary stain; thionin, basic fuchsin, safranin and still other anilin dyes have their adherents; even blue fountain-pen ink can be employed. Try any dyes you have on hand. For counter-staining—a step that can be omitted if desired—neutral red, erythrosin, safranin, basic fuchsin, light green, and Bismarck brown are all popular.

Another widely used technique is Loeffler's alkaline methylen blue. Stain films 1 to 5 min., rinse in water, dry thoroughly, and mount in balsam.

### CULTURING BACTERIA

Much of the thrill of bacteriological work comes with the culturing of your own specimens. A few random observations of living forms, supplemented by a stained slide or so are interesting, but you will learn practically nothing about the science unless you are willing to take the time and trouble involved in the more detailed expedition into the land of the bacteria now about to be described. The joys of growing your own microscopic plants and observing their behavior under various conditions is an experience that all microscopists should have; just as we urged you to make your own slides, so now we suggest that you plant your own bacterial garden. A certain amount of expense for equipment is inevitable, but the investment will prove to be well worth while.

The apparatus needed includes a wire inoculating loop, an inoculating needle, centigrade thermometer, hot-air sterilizer, steam sterilizer, incubator, tubing stand, and minimum numbers of the following items of glassware: one funnel, one flask, and three to six each test tubes and petri dishes. If all these are purchased, the larger pieces will run into a considerable figure, but some of them may be converted from household or scientific objects, and the

incubator and sterilizers may be homemade, according to specifications seen in accompanying illustrations.

**A Hot-air Sterilizer.**—As shown in Fig. 95 this appliance may take the form of any metal drum or tin, circular or rectangular, to be heated over a flame or stove, with some ventilating holes punched in the top and, preferably, with the bottom left in place; access to the interior is gained by cutting out a portion of one wall to make a hinged door. The addition of a thermometer will make this sterilizer more

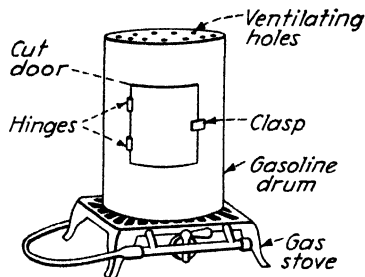


FIG. 95.—Hot-air sterilizer. (From Earl S. Goudey in Bausch & Lomb's "Educational Focus.")

accurate. Cut a circular hole in the top near one corner to take a one-hole rubber stopper, through which the business end of the thermometer may project into the interior. The most desirable temperature is  $160^{\circ}\text{C}$ . It should not be lower if bacteria are to be killed; it may go higher but should not exceed  $200^{\circ}\text{C}$ . Many forms of oven, such as that we have recommended as a slide drier (Fig. 38), may be used for this purpose, and the heat source may be electricity, gas, or oil.

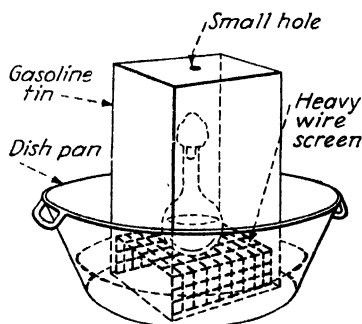


FIG. 96.—Arnold sterilizer. (From Earl S. Goudey in Bausch & Lomb's "Educational Focus.")

**An Arnold Sterilizer.**—The hot-air appliance serves for glassware, but the high temperature is not suitable for metal instruments, nutrient media, or the various cultures of bacteria, which are sterilized at a lower temperature by live steam under pressure. Elaborate and costly professional steam sterilizers, called "autoclaves," are designed

for quantity work in large laboratories; the household pressure cooker may be adapted; or the much simpler Arnold sterilizer either purchased or made at home (Fig. 96).

The principle involved is to provide a flat tray or pan to contain a wide sheet of water of very shallow depth and so produce a quantity of steam quickly when heated, as over a gas stove. A rectangular metal container, such as one of the large tins used for gasoline, motor oil, sirup, and other commodities, forms the sterilizer proper, with the bottom cut out and removed, and a small hole drilled in the top center to serve as a vent.

A second and slightly larger tin—not shown in the illustration—is placed around this to form a condensing jacket. Steam rising from the pan fills the inner tin and maintains a constant temperature of 100° C. under a slight pressure. It then escapes through the vent, cools and condenses to water, and drips back inside the outer jacket to the heating pan again, the same water being used over and over, with occasional small renewals as necessary. The articles to be sterilized are supported above the water level upon some form of small metal table; probably the simplest of all to make is the sort shown in the figure—a double fold of heavy galvanized screening of ½-in. mesh.

**An Incubator.**—The development of bacterial colonies in culture media requires a low-temperature incubator, the source of heat for which may be an alcohol or kerosene lamp, gas flame, electric bulb, or electric heating unit. Many types are on the market, from simple to complex; the commercial poultry incubator may be adapted and the slide drier previously described will serve excellently. Figure 97 shows how a very serviceable model may be constructed at low cost. In all homemade models see that no inflammable materials are placed near the heat source. A frame of hardwood, 18 in. high, 12 in. wide, and 10 in. deep, may be drilled to receive screws by which slabs of sheet rock or asbestos board can be fastened to form a very fine homemade incu-



bator. The front should be a swinging door, with or without a panel of glass. Do not neglect ventilating holes around the top of the sides. Drill the top also to receive a thermometer in a rubber stopper and a thermoregulator, such as is sup-

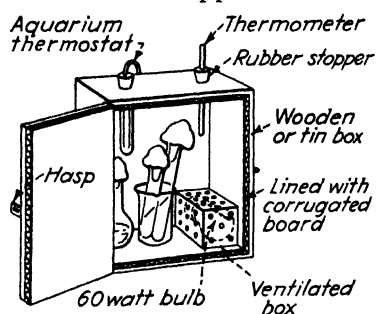


FIG. 97.—Incubator. (From Earl S. Goudy in Bausch & Lomb's "Educational Focus.")

plied for aquaria by dealers in tropical fish. Drill the back, toward the bottom, to receive electric wires, and install either a heating unit or an electric bulb placed horizontally.

On the inside, screw or bolt on wooden cleats on which shelves are to slide. The shelves should be made of heavy, wide-meshed galvanized screening, with the edges crimped or bound, should be perfectly level, and free to slide in and out. One or two of the upper shelves may be removed when large objects, resting on the bottom, are to be incubated.

**Sterilizing and Plugging.**—Clean all glassware first with a 24-hr. bath in bichromate-sulphuric glass-cleaning mixture. After rinsing, place them in the hot-air sterilizer for 1 hr., longer if more convenient to fit your schedule. Test tubes, petri dishes, and such small items can be handled more easily if placed together in a cleaned coffee can or a cylindrical basket made from galvanized screening; many pieces may be inserted or removed with a single operation.

As soon as the test tubes are dry, they are removed and *plugged*, then replaced in the sterilizer. If possible, have a professional bacteriologist, student, interne, or physician show you how to plug a tube properly. If no such assistance is available, proceed by taking two strips of sterile absorbent cotton and laying them crosswise, with a third and smaller piece in the center. Gather this up and force it into the mouth of a test tube for some distance—not less than one-

fourth of the tube length—and leave plenty of the fluffy end protruding. Force it in with the butt end of a pencil or a glass rod, then test by holding on to the exposed end of the cotton to see if the plug will support the weight of a tube half filled with water. The plug should retain its shape when removed. This making of plugs requires practice but it is soon mastered.

Plugged tubes may be sterilized by boiling in a saucepan for 30 min., or heating the dry pan in an oven for 1 hr. at 160° C., or in a double boiler; if much of this work is to be done, we advise the purchase or manufacture of the hot-air sterilizer.

**Culture Media.**—A culture medium is a decoction of some form of food on which bacteria will thrive, and the number of such media is legion. The manufacture of a few of those most widely in use follows:

*Nutrient Broth:* *Method 1*, purchase ready prepared for use. *Method 2*, to 1,000 cc. of distilled water add 10 g. of peptone, 5 g. of Liebig's meat extract, and 5 g. of salt (sodium chloride). Heat until dissolved, then add distilled water up to the 1,000-cc. mark to replace that lost by evaporation. Tube and sterilize, as hereafter directed.

*Nutrient Agar:* *Method 1*, buy ready prepared. *Method 2*, purchase the solid preparation termed "nutrient agar," which has only to be mixed with nutrient broth to be ready for use. Put 20 g. of powdered nutrient agar in a mortar and grind up with a bit of the broth to make a paste. When smooth, add the rest of 1,000 cc. of broth and cook until the agar is all dissolved. Use a double boiler, with a 25% salt solution in the outer vessel, to ensure actual boiling of the contents of the inner. *Method 3*, cook up 15 g. of shredded or powdered agar in 1,000 cc. of distilled water in an agate vessel until thoroughly dissolved (30 to 45 min.). While cooking, add 10 g. of Witte's peptone, 5 g. of Liebig's meat extract, and 5 g. of sodium chloride. When the agar is dissolved, add distilled water to the 1,000-cc. mark to make up

loss, cool to 60° C., and add the whites of two eggs. Heat for 30 min., stir and heat for 15 min. more, then add distilled water again to make up loss. Tube and sterilize.

*Gelatin Medium: Method 1*, obtain in dehydrated form as nutrient gelatin, which is cooked up in distilled water. *Method 2*, dissolve 5 g. of Liebig's meat extract and 5 g. of best French sheet gelatin in 1,000 cc. of distilled water by heat. Add water lost by evaporation, cool to 60° C., and add the whites of two eggs. Stir and heat for 30 min. in an Arnold sterilizer. Replace water lost, tube, and sterilize.

*Potato Slants*: Scrub one or more large Irish potatoes until clean. Peel off a large amount, then using a ½-in cork borer, a piece of brass tubing of ½-in. diameter, or an apple corer, take out some long cylinders through the potato and cut each of them diagonally into long, narrow wedges. Soak these overnight in a 1 % bicarbonate of soda to correct the acidity. Now put a small bit of cotton saturated with distilled water in the bottom of a test tube, and then a potato wedge so as to rest on the cotton, which is to prevent drying out of the potato. Carrot or cucumber (seeds removed) also makes good slants, prepared in the same manner.

**Tubing.**—By tubing a medium is meant the placing of a liquid nutrient in a sterile test tube. This is best accomplished by having a funnel supported in a ring stand, with a section of rubber tubing leading from the funnel and fitted with a clamp (Fig. 98). Insert a medicine dropper barrel in the delivery end of this tube. Plug the neck of the funnel with moist cotton and pour in the freshly prepared broth or agar.

A plugged and sterilized test tube is then picked up with the left hand out of its rack or container and the plug twisted with the right hand until it breaks its seal and rotates. Now, holding the right palm upward, grasp the protruding part of the plug between the bases of the middle and ring fingers and remove the plug. It is held thus through

any subsequent steps and must never be put down or allowed to come in contact with any other object. This method leaves the fingers of the right hand free for other duties. With this hand place the delivery tube from the funnel in the test tube and release the clamp, filling the tube one-third full, but exercising care not to get any of the medium on the sides of the tube. Replace the plug in the tube and proceed to fill the next tube in the same manner.

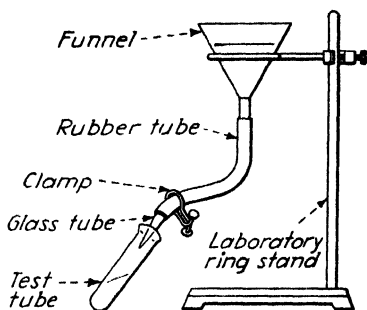


FIG. 98.—Setup for tubing media.  
(From Earl S. Goudey in *Bausch & Lomb's "Educational Focus."*)

Some of these filled tubes may be placed upright in a test-tube rack, but those containing media that solidify at room temperature are inclined so that the medium will set while on a long slant. All media, after being tubed and replugged, are sterilized in an Arnold sterilizer for 30 min. on each of three successive days.

The tubes are then transferred to the incubator and held there for several days at  $37^{\circ}\text{C}$ . to make sure that the media are in fact sterile. If bacteria are present in a fluid medium, they will make their presence known by evidences of putrefaction, including cloudiness, gas bubbles, and foul odor; on solid media they will develop into colonies of visible size, appearing as white or colored specks or streaks, which continue to grow and spread. If no such evidences appear, the media are sterile and ready to inoculate with forms that one desires to culture.

**Inoculating Media.**—From any of the sources for bacteria previously described, such as sewage, hay infusion, or saliva, let us first prepare a mixed culture of saprophytic species—those living on dead organic matter. Pick up a tube of liquid medium in the left hand, the wire loop in the

right. Flame the loop, then remove the plug with the right hand held palm upward, as before. Dip the loop into the culture of bacteria, then flame the mouth of the tube for a few seconds and insert the loop in the medium, without allowing it to touch the sides of the tube or any other object. Remove the loop, flame the mouth of the tube again, replace the cotton plug, flame the loop, then place the tube in the incubator for 24 to 48 hr. A slant of solid medium is similarly inoculated by observing all of these precautions against contamination and by drawing the loop gently along the surface of the slant from the base upward, without breaking the surface.

This exacting routine may seem unnecessary in a case like this, where you are unconcerned as to what kinds of bacteria you are obtaining and wish simply any bacteria at all; it is *very essential* when inoculating specific types to get a pure culture. Each of these steps is necessary to prevent bacteria in the atmosphere, on the tube, or on the loop from gaining access to the broth and thus contaminating the culture with foreign species. It is the regular practice of professional bacteriologists and should be carefully and exactly followed in all cases, for practice and to acquire the routine, and to avoid lapsing into sloppy, unscientific habits.

The right temperature for the incubator will vary with the kind of bacteria one is cultivating. Body temperature, 37° C., is correct for pathogens and for commensals in or on the body of man or mammals; 20 to 30° C. is best for saprophytes. Incubation at room temperature is often sufficient for these. Most bacteria cease to grow at temperatures below 15 or above 45° C., but they are not necessarily killed until much colder or hotter extremes are reached. For each species there is a range of temperature limited by a minimum and a maximum, somewhere between which is an optimum, the temperature at which they thrive best.

**Pure Cultures.**—Inasmuch as a single loopful of fluid from a contaminated source may contain between two and three billion bacteria, representing a number of separate species, it would at first thought seem impossible ever to isolate an individual specimen from such a mass, yet it can be done with ease by either of two methods, known as “plating” and “streaking.” They result in the growing of a colony from a single bacterium.

*Plating:* The required materials are three sterile petri dishes, three tubes of melted agar cooled to 45° C., and an inoculating needle. This last is made of platinum or nichrome wire and differs from the similar loop in shape, the end being pointed. Flame the needle and dip it into a fluid suspension of bacteria to a depth of about  $\frac{1}{2}$  cm. Then, using all the precautions against contamination previously discussed, unplug tube 1, insert the needle in the agar, and rotate it a few times between the fingers, plug the tube, and replace it in its rack, and, without sterilizing the needle or allowing it to touch anything, inoculate tube 2 and then 3 in the same manner. After being plugged, each tube is rotated between the palms of the hands so as to mix the bacteria with the agar. Obviously, by this method, each tube receives a progressively smaller amount of infection.

Taking tube 1 out of its rack, unplug as before, holding it in the right hand. With the left hand, lift up the lid of petri dish 1 on one side and just sufficiently to permit pouring the contents of the tube into the dish. Do this quickly and immediately drop the dish cover back into place. Repeat with tubes 2 and 3, pouring into dishes 2 and 3. To keep these pieces of glassware straight, the numbers should be indicated by paper labels or by a glass-writing pencil of soft wax. Let the dishes cool, then turn them upside down and incubate them in that position.

The first dish will show a heavy infection, the other two progressively much less, with few and more scattered

colonies. Each colony results from the growth of a single bacterium and hence represents a pure culture. From dish 2 or 3 it will not be difficult to locate a colony growing by itself, not mixed in with or too close to some other group, and to pick up a portion of it on the tip of the sterilized needle. Transfer this material to a fresh tube of nutrient medium, where it should grow vigorously upon incubation.

*Streaking:* This method is simpler and preferred by many, though we advise trying both plating and streaking to gain the necessary experience. Here the petri dishes are inoculated rather than the tubes. Use three sets, as before, and pour tubes of melted agar into the dishes, as with the plating method. When these have cooled, a small sterile glass rod or the flamed loop is dipped into broth or other fluid source for bacteria, and stroked across the solidified agar surface of dish 1, in parallel lines about 1 cm. apart, without injuring the surface of the agar. Repeat with dishes 2 and 3, with the same rod or loop, without sterilizing it between times. Colonies spring up along these lines after incubation and are farther apart as one proceeds toward the last line of dish 3; here and there will be pure cultures from which a bit may be taken on the needle for transfer to a broth medium, which is then incubated.

Thus are bacteria obtained and reared.

**Running Down the Type.**—Studying and identifying the many species obtainable by the foregoing methods are other matters which eventually will call for considerable knowledge and experience. Permanent slide preparations should be made and are of many sorts for various purposes. They may be made directly from the original source, without culturing, in order to show the bacteria present, as in pond water, sewage, soil infusion, or saliva; or one may desire a slide to show one species only, made from an incubated pure culture. Still other slides may require special treatment in order to bring out such structural features as spores, capsules, and flagella, and thin sections

of organic tissues from infected animals or plants can demonstrate the pathogens of a given disease as they occur in the body.

Bacteriology texts and manuals will give many characteristics by which the colonies themselves may be identified. The appearance of these groups on different culture media, both liquid and solid, the resistance to heat, toleration of oxygen (aerobes) or inability to grow in an atmosphere containing oxygen (anaerobes), behavior when treated with certain chemicals, and still other tests may be made before recourse is had to any microscopic examination. The two forms of study should supplement one another in determining the species. When you are ready to make stained slides, there are certain methods that readily divide all bacteria into groups according to their reactions to certain dyes, and the presence or absence of definite structures.

1. *Gram Stain*: This famous technique has already been given, and permits a primary division of all species into either Gram-positive or Gram-negative.

2. *Ziehl-Neelsen Acid-fastness and Spore Stain*: This is an excellent general stain for bacteria in either smears or sections. It keeps well and has the additional advantage of staining certain forms, as leprosy, smegma, and tubercle bacilli which resist staining with methylen blue and other dyes. It distinguishes between bacteria that are acid-fast and those that are not, and also demonstrates the presence of spores excellently. Prepare a dried and fixed film as before, then flood the preparation with carbolfuchsin and steam gently over a low flame for 5 min., without boiling. Wash well in running water then decolorize—with either 1% sulphuric acid in water or with 90A to which 3% by volume of hydrochloric acid has been added—until no more color can be extracted, and wash again in water. Counter-stain for 1 min. with Loeffler's methylen blue, wash in water, dry completely, and mount in balsam. Bacteria that do not give up the fuchsin after decolorizing with



acid are termed "acid-fast." These and any spores present will be red; other species blue.

3. *Capsule Stain*: Some bacteria are surrounded by capsules, others are not. To determine this point as an aid in the identification of unknowns, prepare a smear without water and dry in the air. Do not fix by heat, as is usually done. Stain with aqueous gentian violet and heat gently until the stain steams; then wash immediately with a 20% solution of copper sulphate, dry, and mount. It is important to note that no water is used in this technique at any stage.

4. *Flagella Stain*: Bacteria able to move about—motile species—possess from one to many long hair-like flagella, and these require special staining to demonstrate. The stain must have a mordant and is not always successful, sometimes several trials being necessary to get a good slide. Among the best species for this purpose are giant ones occurring in hay infusion or swamp water; among pathogens, the typhoid bacillus is well known as an excellent example, though it should be strictly avoided by the untrained.

*Method 1*: iron-alum mordant. Make, dry, and fix in the customary way several thin films from a young and vigorous culture of motile forms. Pour the mordant on the film for 5 min., gently warming by holding it high above a flame but taking care not to allow it to boil. Rinse in water, then stain faintly with carbolfuchsin. Rinse and examine under the microscope with the highest power available. If successful, dry and mount in balsam; if not, repeat until success is attained.

*Method 2*: tannic acid mordant. Allow several loopfuls of a young culture of suitable bacteria to dry on a slide or cover glass without spreading, then heat-fix as usual. Pour on the mordant and heat gently for 5 min., adding more mordant as necessary to keep the film covered. Wash in running water, dry, add gentian violet, and heat again for 2 to 5 min.; then wash, dry, and mount in balsam.

In general, for all slide work, young cultures, 24 to 48 hr. old, should be used.

**Sections.**—Sectioning methods for tissues containing bacterial infections do not differ from those for other general materials and are considered in Chapters 12–14. Sections should be cut quite thin, not over 5 microns, owing to the extremely small size of these organisms.

One of the best general objects with which to demonstrate bacteria as they appear in place in some organ of the body is the liver or spleen of a mouse which has previously been inoculated with *Bacillus anthracis*, a large rod-shaped species that produces the dangerous disease anthrax. Pieces of such tissue are fixed in Zenker's or Bouin's fluid, fastened to slides with albumen fixative after being cut by the paraffin method, and stained by the foregoing Gram or Ziehl-Neelsen technique.

No one but an experienced bacteriologist should attempt such inoculations or in any other way handle the living anthrax bacilli; the amateur may often obtain fixed bits of organs, which are of course perfectly safe to section and stain since they contain no living organisms. Most readers will be able to contact a hospital or college bacteriologist, advanced student, or city laboratory worker, directly or through friends or the family physician, and thus obtain a sufficient variety of safe materials.

## CHAPTER 10

### MICROSCOPIC SKELETONS

*In This Chapter:* collecting, cleaning, and mounting diatoms, foraminifera, radiolaria, sponge spicules, holothurian anchors and plates, and miscellaneous objects of similar nature.

THERE are certain small plants and animals with skeletal elements composed of either silica or lime, which have always been especially loved by the amateur microscopist, notably by our British cousins, but which for some reason have been neglected of recent years in biological courses in American schools and colleges. They are objects of great interest and beauty, any or all of which comprise a fascinating hobby in themselves. We hope that some of our readers, unfamiliar with them, will be stimulated to try the following procedures.

#### DIATOMS

**Nature and Importance.**—Diatoms are microscopic, unicellular plants (Fig. 99) belonging to the green algae, and are found in both fresh and salt waters all over the earth, in present as well as in past ages. The living cell substance, termed the *protoplast*, secretes a glass box, made of silica (adjective, siliceous) which remains behind and is highly resistant to destruction after the death of the plant. Hence untold billions of these skeletons have accumulated in vast deposits over certain portions of the globe and make up diatomaceous earth or kieselguhr or infusorial earth. One notable deposit in this country is found at Richmond, Va.

Each box or *frustule* consists of two halves, the *valves*, fitting together like the two parts of a pillbox, the line of union being the *girdle*. Under the microscope, the valve view often differs markedly from the girdle view in the same

species, so that both have to be known for purposes of identification. A few diatoms are colonial but most of them are independent. Over 10,000 species have been described, presenting great diversity in shape and especially in the

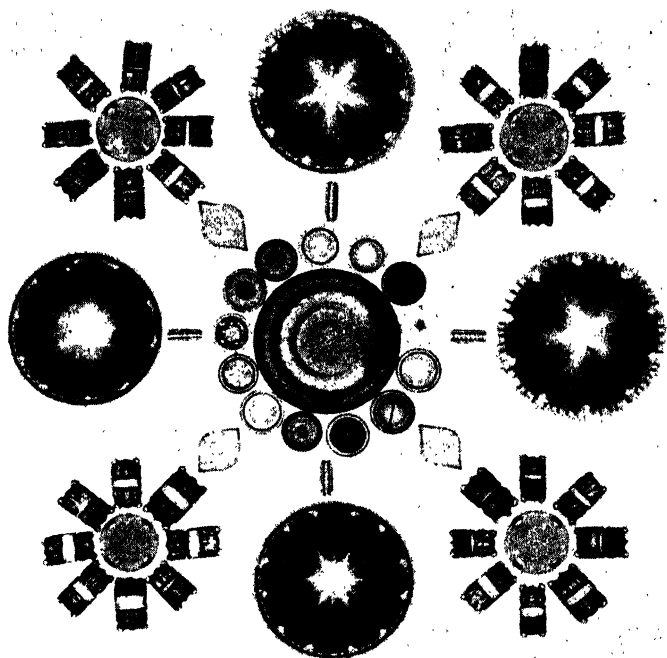


FIG. 99.—Diatom exhibition slide, 68 X. A marvelous example of the microscopist's art and an object of great beauty.

many intricate markings and sculpturings, but all are bilaterally symmetrical and transparent to translucent. By their absorptive and refractive effects on transmitted light rays, many forms appear gorgeously colored with greens and blues; others are colorless. They are among the most beautiful of all natural objects; because of this fact plus their immense number and variety, they have long been eagerly sought by diatomists, humorously dubbed "diatomaniacs."

Diatoms have many commercial uses. Because diatomaceous earth will absorb more fluid per volume than any

other known material, it is a vehicle for nitroglycerin in the manufacture of dynamite. Its porous condition and light weight make it ideal as a packing medium, and it conducts heat so poorly that it finds another use in packings around steam pipes. Being hard and abrasive, it is widely employed as a scouring and polishing medium in the manufacture of soaps, powders, and pastes—the hobbyist may, in fact, secure a good many species by making smears from toothpaste and silver polish. Textile and other designers have drawn inspiration from the endless variety of geometrical patterns exhibited by these tiny shells.

Our own science of microscopy leans heavily on this mighty midget clan. Certain favored species with intricate markings require a very fine objective lens to resolve their pattern, and have become favored the world over as “test objects” by which the magnifying and resolving powers of lenses may be judged, both at the factory and by the purchaser. So hotly have disputes raged in the past as to the exact details of diatom construction that specialists in this field have demanded ever better and more perfect lenses, and should receive at least a share of the credit for the present high state of development of microscope objectives.

Of even greater fundamental importance is the role played by diatoms in the food cycle of living beings. Like all green plants, these minute cells manufacture their food from inorganic materials, and are themselves eaten by a host of small animals, such as protozoans, hydra, worms, rotifers, and microcrustacea. These then serve as food for larger animals—invertebrates, fish fry, and tadpoles—which in turn are eaten by still larger aquatic forms, as bullfrogs, larger fish, and mink, and so on; the cycle ending in man when he eats fish. Thus diatoms constitute one of the largest and most fundamental groups standing at the base of the food pyramid, and synthesize inorganic materials into the organic for an important portion of the earth's inhabitants. Speaking of fish as a nutritious food, a naturalist has said, “All fish are diatoms.”

**Collecting.**—Diatoms are found in all aquatic situations, from the ocean to the smallest roadside ditch, and constitute the largest item of the plankton everywhere; they also exist in enormous numbers on the bottom too, where they cover soil, rocks, submerged timbers, and such other forms of life as the larger algae, corals, and shells.

Bottom dwellers are skimmed by using a tablespoon or a piece of used photographic film and going gently over the surface of underwater objects and mud bottoms. Stalks of algae are scraped into a crystallizing dish; floating species are taken in a plankton net. Diatomaceous earth may occur in your vicinity, as can be determined by inquiry from your county agent, state university, or soil conservation official; if not, then small pieces from various localities can be purchased at slight expense from supply houses. Exchanges constitute an important method of adding to one's collections in this group, inasmuch as there are specialists in every corner of the globe.

**Cleaning.**—As diatoms are ordinarily collected, they are mixed with mud, sand, and organic materials and require a thorough cleaning as a preliminary to mounting. Owing to the indestructible nature of silica, diatom frustules can be put through an amazingly drastic series of chemical procedures, seemingly certain to destroy almost anything, yet emerge unharmed in pristine glory. Each specialist has his own kinks in this work, varying here and there from the program adopted by others. We have found the method given us by H. W. H. Darlaston, Birmingham, England, unexcelled, and herewith reproduce it:

*Living Diatoms:* Put the scrapings, as collected, into a jar of water. On reaching home, strain through coarse muslin to remove the larger stones and soil particles, then put the strained material into a clean jar and fill with water. Allow to settle for 1 hr., then put the jar under a tap and turn on a gentle stream of water, flushing for 12 hr. or overnight. The stream must be very gentle to avoid washing away

the diatoms. At the end, the water in the jar should be perfectly clear, with a fine sediment on the bottom.

Stand the jar in daylight for 6 hr., when the diatoms will be found gathered on the surface of the sediment. Remove them with a pipette and either proceed to clean them at once, or store in 5% formalin if more convenient to do the work later.

When ready to clean, boil the diatom material first in strong hydrochloric acid, using test tubes or other small vessels and small amounts of the acid. Avoid spilling the acid or inhaling the fumes; do the work outdoors or use the fireplace as a chemical hood. Wash in several changes of water by filling the tube, allowing the diatoms to settle, then decant carefully. Repeat two or three times.

Next boil in strong nitric acid and wash in several changes of water; then boil in sulphuric acid and, while still hot, add a few small crystals of potassium chlorate. This will cause a violent effervescence and must be done slowly and cautiously in an open dish, such as a porcelain evaporating dish. Continue to add the chlorate until the effervescing ceases; then wash in several changes of water, as before, and the cleaning is finished.

These complex measures have their reasons. The hydrochloric acid kills the plants and removes all calcium salts, the other acids remove organic matter, and the potassium chlorate bleaches. None of these acids attacks silica. Remember never to pour water into sulphuric acid since the heat thus generated is terrific and might shatter the container. Do not pour acids into household sinks.

*Diatomaceous Earth:* Break up the lumps by stabbing with a screw driver or ice pick, but avoid pulverizing. Put a layer of small pieces of this earth into a saucepan and cover with a layer of crystals of sodium hyposulphite. Heat over a flame until the crystals are melted and the earth fully saturated with the absorbed melted hypo; but do not add water at any time. Allow to cool for 1 hr. or so, then add a few more crystals of dry hypo and heat again

to ensure thorough impregnation of the earth. Then allow to become entirely cold, when the absorbed hypo will have recrystallized and the earth become broken up to its finest particles by expansion of the forming crystals.

Now fill up the saucepan with cold water, producing a muddy mass that will separate in a few minutes into a bottom sediment and a floating scum. The sediment will contain all foreign solid matter and broken diatoms, the scum only perfect diatoms, buoyed up by air trapped within them. Skim off this surface accumulation thoroughly and carefully, as long as it continues to form.

With the remaining cleaning processes, handle the sediment and scum separately. Wash first with water to remove all traces of hypo, then boil for 10 min. in a strong solution of washing soda. Wash in water. Boil for 5 min. in hydrochloric acid, wash, boil for 5 min. in nitric acid, wash, boil for 5 min. in sulphuric acid, bleach in potassium chlorate, and wash thoroughly.

**Mounting.**—The cleaned diatoms may be stored in shell vials of distilled water, a separate vial for each preparation, or may be mounted at once. There are many kinds of mounts for various purposes and also many ways to make any particular mount, as we shall see.

**LOCALITY STREW SLIDE:** Select a vial of cleaned diatom concentrate from any given locality and transfer a single drop to a clean vial half filled with distilled water. Shake and then examine one drop of this dilution on a slide under the microscope to see the amount present. There should be sufficient diatoms so that many are present and well distributed throughout the field, but they should not be so numerous as to form masses and clumps or to lie over and obscure one another. This examination will quickly tell whether to add more diatoms or more water to secure the proper balance.

**Balsam Mount:** Place a thoroughly cleaned cover glass on the table before you and breathe on it; then immediately



add one drop of the diatom suspension, which should spread out into an even thin film. Invert a glass vessel over this cover to protect it from dust while it is drying. When absolutely dry, put a drop of balsam in the center of a very clean slide, invert the cover glass over this, diatom side down, and drop it gently into the balsam. When the slide is dry, clean it, ring on a turntable if preferred—a step done more for beautification of the finished slide than from any necessity—and affix a suitable label, such as, “Diatom Locality Strew—Todd Hollow, Bristol, Conn.—R. F.” The R. F. is a convenient abbreviation from the following list:

R. F.—Recent, fresh water.

F. F.—Fossil, fresh water.

R. B.—Recent, brackish water.

F. B.—Fossil, brackish water.

R. M.—Recent, marine.

F. M.—Fossil, marine.

*Dry Mount, Cover Burned On:* The drying of the cover glass in the preceding balsam mount may be hastened by heat. Many diatomists believe that heat should be so used in all cases, to ensure thorough dryness before mounting. Make a cover glass film, as before, and place this, diatom side up, on a hot plate, or heat over an alcohol lamp or bunsen burner, laying the cover glass on a small piece of platinum foil of about  $\frac{1}{100}$  in. thickness. Bring it to a dull red heat, but no higher. This not only ensures perfect dryness but affixes the specimens to the cover, hence the phrase “burned-on.” Spin a ring of the proper size for the cover glass on a slide and allow it to dry until tacky; then, with both slide and cover warm, invert the cover, diatoms down, and press its edge into the ring all around. When cold, seal by ringing.

In addition to these two methods of mounting, other media are very popular and are necessary with test slides, since the refractive index (R. I.) of balsam is not high enough satisfactorily to resolve the exceedingly fine striations and pores on many diatom shells. Resinous media with a high R. I. are generally preferred for most diatom slides, though balsam or dry mounts are satisfactory with

locality strews, where the purpose is mainly to identify the species present at a given region.

*Styrax Mount*: This resin is widely used, as the R. I. is sufficiently high to resolve most diatoms. Two forms are employed, European styrax (*Liquidambar orientalis*), R. I. 1.582, and American styrax (*L. styraciflua*), R. I. 1.63. The R. I. of Canada balsam, for comparison, is 1.526. Both of the styraxes can be obtained dissolved in xylene, benzene, or one of the alcohols. Johnson recommends chloroform as the best of all solvents for use with diatoms. Mounts are made as specified below for hyrax.

*Hyrax Mount*: This is a synthetic resin, derived from naphthalene, with an R. I. of 1.71, and generally preferred to styrax for diatoms. It is soluble in xylene and benzene, but not in alcohol. Specimens are burned on a cover glass, then treated with a drop of xylene to exclude all air from the valves. Put a drop of hyrax on a slide and invert the cover to mount it; then place the slide on a hot plate at such a temperature that the fingers can tolerate touching the plate for a few seconds only. Bake the slide thus for 2 days, or until any exuded hyrax remains hard. Cool the slide and clean off any excess mountant with a bit of cotton wrapped around a toothpick and moistened in benzene. Return the slide to the hot plate for 24 hr. more, then cool and clean by wiping out of alcohol. Again put the slide on the hot plate and leave it there until convenient to ring with varnish; a week or more of such heating does no harm, but the slide may be ringed whenever desired.

*Sirax* is another synthetic resin with a still higher R. I., 1.8. It is treated the same as for hyrax but has a bad tendency to form troublesome air bubbles.

*Piperine*, R. I. 1.681, is a good mountant as far as its high R. I. goes, but it is apt to crystallize and turn opaque, whereupon it must be heat-treated further to fuse the crystals again. This process may be repeated indefinitely without harm. To mount with piperine, turn on the hot plate in advance so it will be hot when wanted and also

heat the mountant to about 180° C. for 1 hr. before using. Invert a cover glass, with diatoms burned on, over a clean slide and put a drop of the mountant at one edge of the cover so that it will run under; then place the slide on the hot plate. Cool the slide and clean it up, but do not ring as this would interfere with subsequent reheatings.

*Realgar Mounts:* This sulphide of arsenic, realgar, has the highest index of refraction of all mountants, 2.549, and is hence desirable for diatoms with the finest structures; but it is difficult and dangerous to use, owing to its highly poisonous character. There are many methods, that of Grayson being perhaps the simplest and best. He took a small and shallow brass vessel with a loose-fitting lid having a central opening of  $\frac{3}{4}$ -in. diameter. Metallic arsenic and flowers of sulphur, of the finest qualities obtainable, were used to make the realgar. A small amount of the arsenic is placed in the brass container and heated over a bunsen burner; then a few grains of the sulphur are added and a clean slide placed over the opening in the lid. Rising fumes coat the glass, first with sulphur, then with arsenic to make realgar. When the film becomes dark and thick but before it runs into drops, the slide is removed, whereupon the film cools to a lemon-yellow color and exhibits a certain amount of cracking if properly prepared. Then the diatoms are mounted by inverting a cover with the specimens burned on; while still warm, the preparation is ringed thickly with a mixture of gum and shellac. Finished slides should be kept in a dry situation.

It cannot be emphasized too much that this operation is highly dangerous and should not be attempted by the beginner who has had no training in chemistry or in the handling of poisonous materials. The realgar itself is very deadly, likewise the fumes given off during preparation. The work should be done either outdoors in the sun or indoors under a chemical hood, provided with an exhaust fan to draw off vapors. The entire operation can and should be done very quickly.

**SPECIES STREW SLIDE:** The specimens are strewn irregularly over the cover glass but are all of the same species, and designed to present many individuals in all possible views, for intensive study of species characteristics. Material is taken from a vial of concentrate, a separate bottle for each species collected; and since diatoms are seldom encountered in nature other than as mixed species, it is necessary first to isolate separate forms from locality strews. The operator places a drop of cleaned locality strew material on a slide and proceeds to pick up individual cells, under the microscope, and deposit them in the proper receptacle—one of a row of labeled vials containing distilled water—a vial for each species found.

For handling separate diatoms in this way there are many devices, ranging from a moistened, fine-pointed brush of badger's or camel's hair, a cat's whisker mounted in a wooden handle, or a mechanical finger. The older school of diatomists prided themselves on their possession and ability to use dexterously a tiger's whisker for this purpose. A single straight badger hair from a shaving brush makes a good implement. Fasten the base of the hair to a wooden handle with a drop or two of shellac or balsam and when dry, finish with a wrapping of adhesive tape.

**CIRCLE SLIDE:** Selected diatoms, obtained individually by the method just described, are arranged in the form of a small circle, compactly spaced so that the entire group may be seen at one time under a 32-mm. lens. Some technicians prefer to mount the circle on the slide, others on the cover glass. Commercial slides are priced according to the number of specimens thus arranged, standard amounts being 50, 100, 150, and 200. It would be well to purchase one of these, as well as one exhibition slide, to serve as models if you intend trying your hand at these. With the turntable, spin a circle of the desired diameter on the wrong side of the slide or cover, using this as a guide in mounting, then washing it off when the completed slide is dry. Ink is as good as anything for this temporary circle.

**EXHIBITION SLIDE:** See Fig. 99 for an example. The principles of mounting are the same as before, but the diatoms are selected for their size and shape to fit into a predetermined geometrical pattern. The possibilities here are endless and the results remind one of the complicated and fascinating figures seen in a kaleidoscope. The operator first determines the approximate number of diatoms he wishes to weave into his scheme—standard units being 50, 100, etc., in multiples of 50—then sketches his diagram on a sheet of cardboard which is placed on the table before him to serve as a guide during mounting. A motif must be selected, which is then repeated throughout the radiating or intersecting divisions, and diatoms are marked by a symbol on the chart, placed so that circular forms mark termini or intersections, elongate types the radii, triangular species at triradii, and so on.

For both circle and exhibition slides, coat the selected space with gum tragacanth, and as each specimen is transferred by the whisker from vial to slide (or cover glass), breathe on the gum to moisten it. The quick drying following evaporation of this slight moisture sets the diatom in the gum.

Both of these types of slides are among the most beautiful of all microscopic mounts, and the accomplishment of a good one is an achievement that gives one a great deal of pleasure and satisfaction. They are splendid as exhibits for one's friends, at a club meeting, or for public display, and project marvelously through a low power microprojector. The work is slow and painstaking and requires only patience and a steady hand; it is by no means beyond the powers of the average amateur if he has some form of optical assistance, such as a dissecting or binocular microscope or lens mounted in a holder so that he can see his work enlarged and yet have both hands free.

**TYPE SLIDES:** These are slides that incorporate one or more rows of accurately identified species, laid side by side, and accompanied by a key list on a slip of paper, giving

names and localities of the diatoms shown, in sequence from left to right as seen under the microscope. Placing of specimens on these and the following slides is done by the same methods already discussed, and the novice would do well to purchase one of each to see what a good example should be like. Type slides are for purposes of identification—to learn species.

**TEST SLIDES:** Only certain species, well known for the intricacy of their markings, are used for these slides, and only perfect specimens. The diatoms may be all of one species, or mixed, in which case they are aligned as for a type slide and a list for identification accompanies the slide. Research experts in optics and related fields, when ordering one of these mounts with which to test objectives, usually state the preferred mounting medium, generally one of high refractive index.

#### FORAMINIFERA

**Nature and Occurrence.**—Second only to diatoms in general interest are the forams (Fig. 100), as members of this group are commonly dubbed by way of abbreviating the longer name. They are large protozoans of the class Sarcodina—the same to which the well-known amoeba belongs—and secrete complex shells, occasionally of silica but mostly calcareous, composed of calcium carbonate or lime.

Living forams, large enough to be visible without magnification, form a part of the ocean plankton or crawl about over the sea floor by means of their fine, long, and branching processes of protoplasm that protrude through the aperture of the shell and through minute pores (foramina), a feature on which the name of the order is based. When life has departed, the shells accumulate in vast deposits termed “ooze” which may be compressed through long ages into various types of sedimentary rock, fossil deposits of forams being well developed in certain regions.

Thus the chalk cliffs of England are one example, and

the Great Pyramid of Cheops at Gizeh, Egypt, another. Forams have their economic value, too. Aside from the use of chalk, now more generally replaced by the mineral gypsum, and of foraminiferal limestones as building stones,

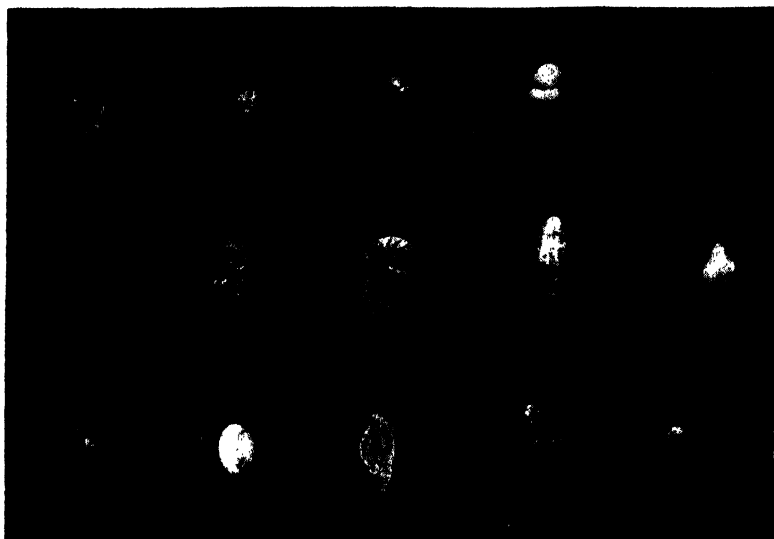


FIG. 100.—Type slide of 15 genera of foraminifera, dry-opaque mount in cell, vertical illumination, 10 X. (*Courtesy of Nature Magazine.*)

these tiny protozoans have come into prominence in recent years as indicators of oil-bearing strata of rocks. Examination of cores from well drillings, under the microscope, apprises the oil geologist of his bearings in relation to the strata sought.

**Cleaning.**—Unless you happen to live in a suitable locality, attempts at collecting will hardly pay and, at least at first, it will be better to buy sand concentrates from a supply house or dealer or secure them by exchange. Being calcareous, the shells cannot be cleaned with acids; instead, bases are used, bicarbonate of soda being a heavy favorite. Johnson prepares material by first breaking up chalk into pieces the size of an olive, then gently crushing these into

a powder fine enough to pass a somewhat wide-meshed sieve. Tie the powder into a bag, immerse in water, and knead until reduced to a third of the original bulk. The resultant milky fluid is poured off, the bag refilled with water, and the mass kneaded again, this process continuing until water coming from the bag is practically clear. Then boil in a strong solution of bicarbonate of soda for 1 to 2 hr., wash in water to remove the soda, and bottle the resulting forams in distilled water.

**Mounting.**—Dry mounts of forams in cells have been discussed in Chapter 6, with or without black backgrounds, or by the half-and-half method. Type and exhibition slides may be made in this manner too. Balsam mounts are frequently desirable; before they can be made, the specimens must be soaked in a clearer until the fluid has forced out all air trapped within the many chambers and pores. Xylene is used for this by some, turpentine by others. Oil of cloves or thin cedar oil may be tried in obstinate cases and sometimes a vacuum pump is necessary; lacking such equipment, one had best reject all material that shows up black from contained air and save only clear individuals for mounting.

Take forams from the bottle of distilled water with a pipette and heat on a slide until absolutely dry, then jar them into a stender of xylene. After soaking, pick up those having no trapped air with a bristle, brush, or pipette, and transfer for mounting to a drop of balsam on a slide. Cover with an inverted tumbler for one or more days until the balsam has partially hardened; then warm, add one drop of thin balsam, and cover, using props.

Some technicians prefer to mount their forams on the cover glass. Warm the cover containing forams in thickened balsam on a hot plate, on which a blank slide is also being warmed. Apply one drop of thin balsam to the cover, pick up the slide and lower it gently over the cover to make contact, then invert. Press the cover down carefully until



the mount is flat, or place the slide on a hot plate and allow the cover to settle by its own weight. Cover props will have to be placed on the cover in the first place, by this method, instead of on the slide. Ring the slide when dry, if preferred.

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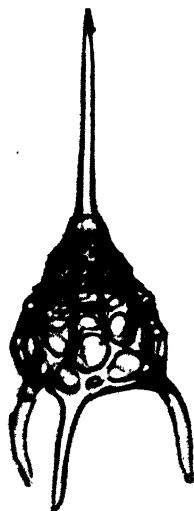


FIG. 101.—*Podocyrthis*, a radiolarian, 148 X.

### RADIOLARIA

**Nature and Occurrence.**—These, too, are protozoa, members of the class Sarcodina, and entirely marine. They are not so abundant as forams and have no commercial value, but they are objects of even greater beauty and delicacy under the microscope (Fig. 101) especially with dark-field illumination. The shells are siliceous, very frail and dainty, and sculptured in most amazing variety. Barbadoes earth is a well-known fossil deposit, a very fine white “sand” made up almost entirely of radiolarian shells. The average individual will have no opportunity to collect material but may purchase small capsules at modest cost.

**Cleaning.**—Radiolaria may be handled more or less like diatoms, but the following method, from Johnson, is to be preferred as less drastic. Break the lump of earth into small fragments, meanwhile boiling 3 or 4 oz. of ordinary washing soda (sal soda) in a pint of water. Boil the earth in this for 30 min. Decant about nine-tenths of this water into a large glass vessel and gently crush the remaining mass. Add soda and water as before, boil again, then decant nine-tenths as before, and continue this procedure until the earth is well broken up. The radiolaria meanwhile have floated off in the several decanted portions and are in the large glass vessel. Stir this and allow to settle well. As the specimens

will be found on the bottom, the liquid above them may be poured off and discarded.

It is generally necessary to take the mass of radiolaria thus obtained and go through the whole process again, from the beginning. When the second set of boilings and decantings has been completed, the sediment is put in a jar with about  $\frac{1}{2}$  oz. of water to which  $\frac{1}{2}$  teaspoonful of bicarbonate of soda is added. After this dissolves, slowly and carefully add 1 oz. of strong C. P. (chemically pure) sulphuric acid. This treatment liberates the individual shells and leaves them beautifully transparent. Wash thoroughly in water to remove all traces of acid and soda and bottle in distilled water.

**Mounting.**—Dry some of the shells on a slide over an alcohol lamp, then jar them into a drop of balsam on another slide or onto a cover glass. Continue as in mounting foraminifera. Another method to ensure an even distribution of specimens throughout the balsam is to take up some of the dried radiolaria on the tip of a penknife blade and, holding this over the balsamed slide, tap the knife gently with any metal instrument. Or one may pipette a drop of the water containing radiolaria onto a slide or cover and warm on a hot plate until fully dry, then add the balsam. Type, circle, and exhibition slides are made as with diatoms.

#### SPONGE SPICULES

**Nature and Occurrence.**—These are the skeletal elements of sponges and are found imbedded in the body wall, serving as anchoring hard parts for the otherwise flimsy tissues. They occur in two forms, calcareous and siliceous, and in several shapes: the monaxon is a long and straight or but slightly curved needle; the triaxon, tetraaxon, and hexaxon spicules are groups cemented into three-, four-, or six-rayed types, modified in some species to include shapes resembling a tuning fork, anchor, or double-ended anchor. Like protozoan shells or diatoms, these resist decay

and are found as minute scattered remnants in the fossil state or in present-day ooze. In addition, many sponges have a framework of horny spongin fibers, with which we are here not concerned.

**Cleaning and Mounting.**—Calcareous spicules are cleaned with a base, siliceous ones with an acid; hence it is best to know something of the nature and classification of sponges and the type of spicule possessed by each form collected. If in doubt, take a small portion of your material and boil it in strong nitric acid then wash in several changes of water, the spicules settling between washings and the supernatant liquid poured off. This treatment will clean siliceous but completely destroy calcareous spicules. With another piece of the sponge in question, use 10% potassium hydroxide, boil, and wash through several changes of water. This will clean the calcareous types but, although not destructive to, is insufficient for the siliceous.

Fresh or dried sponges may be rotted in water until thoroughly saturated, then cut into small pieces for spicule preparation by the above methods. Noll prefers javelle water for this work. Mounting is performed as with radiolaria, and we have seen some very attractive type and circle slides made with the more intricately designed spicules, as well as exhibition slides in which these structures were combined with one or more of the other shells or parts covered in this chapter.

#### HOLOTHURIAN ANCHORS AND PLATES

**Nature and Occurrence.**—The sea cucumbers, of the class Holothuria, are peculiar members of the starfish phylum, elongate and sac-like with little apparent resemblance to the five-rayed starfish plan of construction. All are marine and examples frequently may be collected in pools left along the shore at low tide. Synapta is the genus favored for the present work. In the body wall of this and other types are microscopic skeletal elements of a most curious and interesting nature, looking for all the world

like minute ship's anchors; others resemble perforated plates.

**Cleaning and Mounting.**—Kill a specimen by immersion in equal parts of sea water and ether or chloroform. Probably most readers will have to secure a preserved specimen by purchase or exchange. In either case, cut the body wall into small pieces and boil these in 5% caustic potash, as with calcareous sponge spicules. Mounting of the anchors and plates is done by the method given for radiolaria.

### MISCELLANEOUS OBJECTS

The radula of snails, already considered in Chapter 5, might well be included among the present assemblage. On the upper surface of starfishes occur curious pincer-like organs, the *pedicellariae*, which serve to keep hydroids, algae, and other organisms from growing over the surface and obscuring the gills. These may be cleaned by boiling in 5% caustic potash, washing well in water, and mounting by dehydration and clearing, or by drying.

Insect eggs (Fig. 102) are frequently objects of great beauty under magnification, particularly those with sculptured calcareous shells, as among many of the butterflies. They may be mounted dry or in balsam, but often give trouble as balsam mounts because of trapped air. Try pricking each egg with a needle, choosing a site for the puncture that will not be visible in the finished mount. Make the dehydration thorough and the soaking in clearer prolonged. Use oil of cloves if xylene refuses to clear out all the contained air.

In summary, do not overlook the possibilities of combining two or more of the several kinds of objects taken up in this chapter when trying your hand at circle and exhibition slides. A holothurian anchor is splendid as the terminus of each radius in a geometrical design; an insect egg may form the center or the base of each arm; brilliant but tiny wing covers (elytra) of certain very small beetles, notably the Brazilian diamond beetle, will add flashing colors along the

spokes, and forams, radiolaria, and diatoms may be selected for repeated members of the design. Such slides have no educational value but are worth while from standpoints of beauty as well as examples of the mounter's skill.



FIG. 102.—Arranged group of butterfly and moth eggs, 9.6 X. Photomacrograph by reflected light.

They photograph and project exquisitely, but a word of caution should be inserted about projecting arranged slides. The heat of the beam of light used may melt the mountant and cause the specimens to drift out of position. Beware of ruining a fine slide in this manner and undoing the patient work of hours in an instant. Set up the micro-projector to receive the slide in a flat position, as is done with living cultures, using a mirror or prism to alter the beam from vertical to horizontal. Use cooling cells of water to absorb much of the heat, and do not keep such a slide on the projector for very long at one time.

## CHAPTER II

### GRINDING HARD OBJECTS

*In This Chapter:* making permanent mounts of bone, teeth, rocks, minerals, fossils, and metals.

A NUMBER of miscellaneous substances that cannot be treated in the more ordinary ways are considered in this chapter: tooth and bone, thin sections of rocks, minerals, and fossils, and polished and etched specimens of metals for opaque examination. Although differing greatly in nature, these materials have one thing in common as regards their preparation for microscopical study: they require grinding. This technique is slow and laborious, though the results amply justify the time and efforts expended.

The general method is to saw or chip sections as thinly as possible and then to place these sections between hones or in some other way grind them down to such exceeding thinness that they will readily transmit light and may be mounted as translucent objects. Metals will of course never reach this condition and are always mounted for observation solely by reflected light. One general caution may be given here: as sections are rendered thinner and thinner with continued grinding, they become increasingly fragile and must be handled carefully with a fine forceps, avoiding any unequal pressures or strains that would shatter them.

**Bone.**—Two methods exist for mounting bone. One is termed “decalcification,” by which the mineral content is removed chemically so that the organic material is softened and the bone may then be cut on a microtome, like other tissues. This will be taken up in Chapter 16. The other

method, now to be considered, results in a preparation termed "dry ground bone," and preserves the mineral matter while destroying and removing the organic. Both methods give a good picture of the construction of bone; the two supplement one another and both are necessary to gain a complete knowledge of the subject.

Whenever opportunity offers to secure such an animal as a cat, dog, or rabbit for the fixing of numerous tissues, as taken up in Chapters 12 to 14, remove a thigh bone (femur), clamp it, while still fresh, in a vise, and, with as fine a hack saw as is available, cut several cross sections as thinly as possible, also several longitudinal sections. If no vise is at hand, have someone else hold the bone for you while you cut. A jig saw or any fine-toothed power-driven cutter is even better.

Place these pieces in a dish of water to macerate (rot) until all flesh, grease, and ligaments are removed and the bone is clean. This may take from a week to a month. The water should be changed occasionally; brushing the pieces with an old toothbrush will hasten the process, which takes place through the action of bacteria.

Dry a piece of cleaned bone and place it between two hones, grinding with a circular motion and taking care to keep the hones parallel so as to avoid wedge-shaped sections. Continue until the piece is so thin that fine print can be seen through it easily. Handling the fragile section carefully, wash it thoroughly by flooding with water from a pipette or washing bottle; then put it in absolute alcohol for 15 min. and finally in ether for 30 min. Remove and place it between two slides, with the ends tied with thread or fastened by clips or rubber bands. This will assure drying without warping. When completely dry, the section is ready to mount.

Other methods of grinding are numerous. If a grindstone, emery, or carborundum wheel is available, the rougher work may be done much more quickly. Some technicians use a file for the first thinning, followed by garnet or emery

paper affixed to two wooden blocks, and finish with two hones. Any system of grinding will serve, as long as the two faces of the bone are kept parallel and the final stages are done with something as fine as a good hone, which will not leave scratches.



FIG. 103.—Dry ground bone, photomicrograph, 210 X.

Two methods of mounting are in use, the trick being to get balsam thick enough so that it cannot penetrate the various fine canals that ramify through the bony substance. These are to be left filled with trapped air which, because of the great difference in refractive index between air and balsam, appears black and hence contrasts very sharply with the white bone. The picture presented in a successful slide is a beautiful one of black canals silhouetted against a white background (Fig. 103).

The first method uses thick xylene balsam, which may be prepared from your stock simply by leaving a small quantity exposed to the air in a dust-free situation until enough xylene has evaporated to give the balsam the consistency of thick molasses. Or, more economically, place a



couple of drops of regular xylene balsam on a slide and warm gently and carefully for a few minutes to drive off the xylene. Add the bone section and, with the eraser end of a pencil, a glass rod, or any blunt implement of similar nature, press the bone down into the balsam as far as it will go, then add the cover. In the second method a piece of solid, natural balsam is placed on a slide and warmed carefully until it melts, when the bone section is mounted as in the first case.

**Tooth.**—Sections of teeth and such hard objects as a coconut shell, walnut, and other nut shells, pits of fruits, whalebone, nail, or hoof are prepared in the same manner as bone. Teeth are more difficult to saw than bones; if a very fine saw is not at hand, use the grinding method from the start or make your own saw by the following method: cut a 3- to 4-in. length of very thin spring or band steel, as from an old clock spring or steel tape; or use sheet tin as from a coffee-can lid, though tin is somewhat inferior to steel. Mount in a handle if preferred. Saw across the tooth with a hack saw first to make an impression; then use the steel or tin band, keeping the tooth well wet with water in which is placed a quantity of fine emery powder, fine carborundum, diamantine, or powdered rouge, which acts as the abrasive and does the cutting.

Still another method recommended for this class of object is to saw as thin a section as possible first; then place it on a slab of plate glass together with some water filled with one of these fine abrasive powders. Place the finger or the eraser end of a pencil on the section and rub with a rotary motion until one side is polished smoothly; then reverse the section and grind the other side. When the section has become very thin, cap the first plate glass with another piece and continue to grind and polish by rubbing the top sheet on the bottom one, in rotary fashion. Small pieces of plate glass suitable for this work can be reclaimed from broken automobile windshields and are usually to be

had for the asking at repair shops. They need only to have their edges shaped and ground and to be washed thoroughly.

**Hard Rocks and Minerals.**—Preparation of sections of these hard objects is somewhat similar in method to that used for bone, though the preliminary cutting is more difficult and laborious without special equipment. An old sewing machine may be rigged up by substituting a thin plate of soft iron for the flywheel, plus some sort of clamp to hold the rock specimen while cutting. Any form of lathe can be adapted. It is not difficult to hook up a shaft to a small motor and thus get revolution for a horizontal iron plate, mounted within a box or trough to take care of the mess created. Slices are cut wet, the abrasive being diamond dust by preference, though carborundum will serve. If a water faucet is available to deliver a slow trickle during the work, so much the better.

After cutting as thin a slice of rock as possible in this way, the two surfaces are ground, preferably by machine, otherwise by hand. Steel plates make the most durable grinding surfaces, though plate glass is entirely satisfactory. For very hard and fine-grained rocks, a coarse carborundum or emery powder may be employed first, followed by medium and then fine. With other rocks, omit the coarse and start with the medium. Rub with a rotary motion.

Another method of obtaining specimens for slide preparations is to chip off thin flakes with a cold chisel and hammer, then grind as just described until fairly thin. In either case, most technicians prefer to mount the section or flake before the final grinding. To do so, place a blank slide and the rock slice on a hot plate where the temperature can be somewhat controlled. Start at 50° C. and slowly increase the heat, either by moving the bunsen burner or lamp under the hot plate, or by placing a screw clamp on the rubber tubing of the burner, gradually admitting a greater volume of gas. At 60° C. put some balsam on the slide and some 10 to 15 min. later, with a temperature in the neighborhood

of 115° C., test the balsam by drawing out a thin thread of it with a fine forceps. The balsam is not ready until such a thread is brittle, which will occur between 115° and 120° C., whereupon the rock section is mounted by pressing it down firmly into the balsam; then put the slide away to cool and harden.

Grinding now continues, with the section mounted in this temporary manner, and must continue until the slice is exceedingly thin. It should be tested from time to time under the microscope; if intended for use with a polarizing microscope, it should be tested with crossed nicols until satisfactory. Increasingly fine abrasives are used as the work progresses. When completed, the slide is washed well and then dried. Heat until the balsam melts, then with an orangewood stick or a matchstick that has been sharpened to a chisel edge, gently and carefully push the rock section off of the temporary slide and into some fresh balsam on a clean slide for final mounting, governed by the same temperature regulations as given for the first mounting. The cover glass should be heated also before applying it to the balsamed section, and should be pressed down evenly and carefully.

Between each of the grinding operations, it is important to wash the specimen and scrub the hands well with soap and water. This will prevent carrying over coarse abrasive particles to mixtures of finer ones, and getting any of them into the final mounting. An extra step, which will assist with this point, is to push the finished section off the temporary slide mount into xylene and let it soak until freed of all balsam, to avoid carrying over any of the old balsam; then transfer it to fresh xylene for a short time and proceed with the final mounting.

**Soft Rocks, Coals, Fossils.**—The foregoing methods apply to hard rocks. When soft, friable, or porous rocks, coals and other fossils, or porous fossiliferous rocks are dealt with, the material needs reinforcing in order not to

go to pieces during the grinding, especially in the later stages. After the sawing, sections are soaked in xylene to impregnate them and fill up all spaces, then transferred to a mixture of xylene and balsam which is gradually heated to drive off the xylene. Some workers add a little shellac to the mixture; still others find that imbedding in plaster of Paris before impregnation works well. Chamberlain (*Methods in Plant Histology*) gives two interesting procedures known as *peels* for the taking off of a thin layer of organic material from petrified fossils onto a thin sheet of celloidin or gelatin, which is then mounted on a slide.

**Metals.**—The beginner in microscopy is apt to think of metallography as a study too difficult and advanced to be included in his program; this is not the case. All amateur microscopists during their earlier experiences should make one or two metallic preparations in order to learn the method and understand the results. As mentioned before, such mounts are viewed by reflected light only, and while detailed study with high power requires a vertical illuminator and an objective corrected for use without cover glass, any more or less makeshift vertical or somewhat oblique overhead illumination will serve with the low power objective.

No cover glasses are used, since these would merely cause high lights and glares to no purpose. The size of preparation does not matter particularly, nor the thickness but, inasmuch as one size is as good as another for the amateur, he may as well conform to a widely used professional standard:  $\frac{1}{2}$  by  $\frac{1}{2}$  by  $\frac{3}{8}$  in. in thickness. Such pieces are obtained with a hack saw or other cutting device from various samples of metals, such as iron and copper and from such alloys as steels and brass. The irons among meteorites are also prepared in the same manner. Machine shops and garages can be called upon for specimens as well as for assistance in the preliminary cutting.

When cut to proper dimensions, the metal square is

clamped in a vise and the surface to be treated is ground plane and smooth, with a fine file or a series of files of increasing fineness. Do all of this work in one direction only which, for convenience of description, let us call north-south. Now set the metal firmly into a block of wood, of any convenient size to facilitate handling, with about  $\frac{1}{8}$  in. of the chosen surface protruding. With a file, bevel the edges to a 45-deg. angle.

Grinding and polishing now ensue. Here one important difference from previously described techniques is to be noted: the direction of rubbing is in one way only, never rotary, and is altered at right angles to the preceding rubbing. Thus, if the first filing was done in the north-south direction, the specimen is next rubbed east-west on coarse emery or other abrasive paper, then north-south on paper of the next grade of fineness, and so on. Examine under low magnification at each change to see that all scratches from the preceding rubbing have been eliminated before going on to the next. Stretch the abrasive papers tightly. Preferably they should be cemented firmly to a perfectly flat and hard surface, as hardwood, plate glass, or a steel plate. Decrease the hand pressure as you pass to the finer papers.

When work with the finest abrasive paper has been completed, the specimen is next polished with coarse rouge. For this purpose, stretch a piece of clean damp chamois over a block of wood and thumbtack to hold it securely. Rub the specimen over this chamois, on which the rouge has been placed, in one direction only and opposite to that used with the last emery paper. Wash the specimen and chamois well to eliminate the coarse rouge particles; then repeat with finest rouge or alumina, again changing the direction. The specimen, when washed, should now be perfectly smooth, bright, and shining, like a mirror. When dried, it is ready for etching.

Inspection of the polished surface under the microscope will reveal no structural details, etching with acids or

alkalies being necessary to bring different parts into contrast with one another. Some crystals are attacked more than others; this brings their boundaries into relief and causes scatter of light rays and hence gives a picture of the



FIG. 104.—Polished and etched section of tempered steel, 300 X. Black needles are martensite; white needles are austenite.

components present that would be utterly hidden in the merely polished surface. Etching a metal surface is like developing a photographic film, to bring out what is there but hidden (Fig. 104).

Remove the metal square from its wooden holder and handle by the sides only, with a forceps. Do not touch the polished surface with the fingers or with any implements. Dip into an etching solution, of which any of the following are in general use: for iron and steel, 10% solution of either nitric, hydrochloric, chromic, or picric acid; for copper

and brass or other alloys of copper, 50% ammonia in water or 10% caustic potash. From 15 to 20 sec. are recommended as the time of immersion, then wash quickly in distilled water, swirling the specimen about to remove all traces of the etching fluid. Examine it to see if it has been sufficiently etched; if not, repeat until satisfactory.

For examination, specimens are mounted on slides, fastened by means of plasticene. In order to be sure that an etched metal block will lie perfectly horizontal and true on the slide, place it first face down on an absolutely clean and polished piece of plate glass. Surround it with a metal or glass ring, just a trifle higher than the thickness of the object, or use two wooden blocks, one on each side, as long as they are exactly equal in height (*i.e.*, cut from the same piece of polished hardwood) and protrude slightly above the metal block.

A small quantity of plasticene is now placed in the center of a clean slide, which is then inverted over the metal object and pressed down firmly. Lift the slide up, carrying the object with it. For storage, to prevent rusting, use a desiccator or keep the specimens in a glass jar or metal box along with a container of some calcium oxide.

To grind and etch small metal objects, such as filings and wires, put a glass plate over a magnet and add a small metal or wooden mold on top. Place the metal objects inside the mold and arrange them as desired; then pour in molten Wood's metal, the magnet serving to hold the specimens in place. When the Wood's metal has cooled, the mold is removed and the metal block containing the objects is processed as previously described.

## CHAPTER 12

### SECTIONING: MANUAL AND FREEZING METHODS

*In This Chapter:* freehand, well microtome, and machine microtome methods of sectioning; construction and operation of microtomes; manual and freezing sectioning techniques.

AFTER the microscopist has learned how to prepare whole mounts and smears of a variety of types, he is ready to take the next step and make sections. Most microscopical work in the natural sciences (and much of it also in the applied or the industrial field) is performed from sections; in fact, some 90% of all slides in any general collection will be sectioned materials, including those made by grinding operations covered in the preceding chapter, which are, strictly speaking, sections.

If you were to inspect a large slide collection, you would find sections of such whole animals as hydra, earthworm, and amphioxus; plant parts including root, stem, leaf, flower, and fruit; and animal organs as stomach, liver, heart, kidney, and brain. The profoundly significant events dealt with in embryology and genetics are likewise determined mainly from sections; the research student even requires sections of such minute or thin objects as protozoa, hairs, textile fibers, and papers. The great mass of literature in microtechnique deals principally with methods of fixing, sectioning, and staining as applied to sectioned material.

The preliminaries to sectioning proper are the same in general as those for whole mounts of soft organisms: killing, fixing, hardening, dehydrating, and clearing; however, time durations are shorter for small pieces of easily permeable tissue or hollow organs than for whole animals. After clearing, the next step is infiltration, which replaces the clearer



with an imbedding medium, something soft enough to cut readily yet hard enough to act as a support so that the tissue is reinforced during the actual cutting, and neither crushed nor distorted. After infiltration of some duration, the material is finally imbedded in the same medium and is then cut into very thin slices, which are placed on slides and carried through a more or less elaborate series of staining operations according to the end in view. Finally each slide is dehydrated, cleared, and mounted in the usual way. Sectioning methods will be explained first and then some typical schedules given for the complete performance at the end of Chapter 14.

#### FREEHAND METHOD

Before the advent of machine methods, the only way to section material was the freehand method, by which dexterity with a razor was mastered to the point that very thin, and uniformly thin, pieces of tissue could be cut. Regardless of how much equipment one may have, this technique should be learned; it requires merely a bit of practice, is frequently more useful for immediate examination than the very long and time-consuming machine operations, and gives a certain type of training in manual skill that is desirable.

Either a regulation folding razor (Fig. 53), or a safety-razor blade in a folding holder (Fig. 54) may be used, the latter having the advantage of doing away with frequent and tedious jobs of honing and stropping. The razor is held by the right fist, the specimen to be cut by the left, with the left forefinger held out at a right angle and toward the operator's body. The elbows are resting on the table, as some prefer; if no table is used, they are pressed in tightly to the body. Sections are cut wet, and the action of the razor is that of paring, not that of whittling. For example: take a piece of beef liver that has been previously hardened in formalin or alcohol and hold it between the thumb and forefinger of the left hand, the left forefinger flat and

pointing toward the body. Bring the razor down beyond the specimen, blade flat, edge toward the body; then make a long sliding, slicing cut, the blade entering the liver near the heel of the razor and coming out near the toe. In other

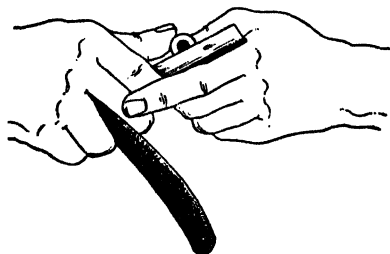


FIG. 105.—Method of cutting freehand sections.

words, the razor is not just pushed flat through the liver; the action is slicing, so that the whole length of the blade is used. Cutting is against the right thumb. If anyone is so awkward with tools that he is apt to include a piece of his own thumb along with the beef liver, then he badly needs this technique to acquire a bit of general dexterity (Fig. 105).

The razor is kept wet by dipping it into a dish of water or by applying water with a camel's-hair brush. As each section is cut, dump it into a vessel of water, or float it off by using the brush; do not touch or handle the sections with the fingers or any hard materials at any time. Make a number of sections for a certain period each day until you can prepare very thin ones with ease, remembering that practice makes perfect. Be careful to avoid wedges; have the sections as thin in one place as in another. Then select a few of the best and thinnest for mounting on slides, stain in hematoxylin, counterstain in eosin, dehydrate, clear, and mount in balsam. Label the preparation as a freehand section and reserve it to compare with a similar machined preparation later on. If only an immediate and temporary examination is wanted, mount the stained section in glycerin.

It is well to remember that the thinnest section is not always the best; all depends on the purpose for which the section is wanted. Sometimes a thick section of an organ, like the kidney, will reveal the general plan of construction far better than a thin one, good only for details.

Small objects are commonly imbedded in pieces of hardened liver, or in pith, turnip, or carrot, and the whole cut as one object; the surrounding material then easily separates from the object when placed in water. For this purpose, secure some strips of beef liver about 2 by 1 by 1 in. in size, place in 95A for 24 hr.; then transfer to fresh 95A and store until needed.

#### WELL MICROTOME OR HAND MICROTOME

After some facility has been gained in freehand sectioning, the student of microtechnique should graduate to the simplest type of machine for this purpose, the well or hand microtome, so designated because it is held in the hand, while the material to be cut is placed in a tube or well (Fig. 106). The principle is merely to have some device that will raise the floor of the well for a known and very gradual distance so that one section may be cut after another and all will be of the same thickness. The idea of the screw at once suggests itself. The simplest and crudest type can be made by grinding the top surface of a large nut very flat and polishing until quite smooth; do the same for the end of the bolt that fits this nut, choosing one with the maximum number of threads per inch. If the bolt is started in the nut and then material is packed into the cavity of the nut, which forms the well of this microtome, it is obvious that a turn of the bolt through one revolution will raise the material through a distance corresponding to the pitch of the screw. If whatever protrudes is now sliced off and another revolution given the bolt, another similar cut may be made, and so on.

All well microtomes are no more than refinements of this scheme, and the manufacture of a homemade hand micro-

tome (Fig. 107) is not difficult, the appearance and utility of the finished product corresponding to the maker's skill with tools. A piece of bakelite or plate glass makes a very smooth flat top or cutting plate which, after a central aper-

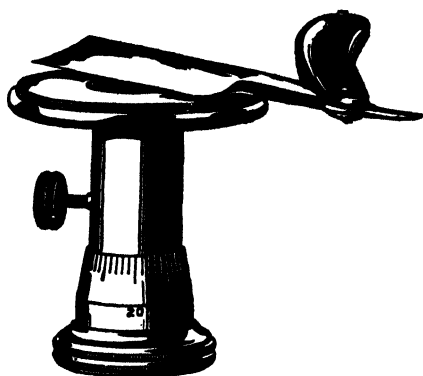


FIG. 106.—Commercial form of well microtome. (C. Reichert.)

ture has been cut out, may be forced upon a brass tube of fairly wide diameter, within which a plunger worked by a threaded screw of fine pitch operates to raise the material being sectioned. In the illustration of a commercial hand microtome (Fig. 106), the side screw is for the purpose of tightening the contents of the well, but is not necessary in homemade appliances.

To use, tissue that has been killed, fixed, and hardened in the regular manner is placed in the well, surrounded by diagonally cut slabs and wedges of reinforcer, such as liver, pith, or carrot so as to make a tight fit. It is important that the object and these other materials reach to the bottom of the well, else they would not be pushed up when the screw is turned. Use a razor exactly as in freehand cutting, drawing the knife across the top plate toward you, keeping the blade flat and using a slicing motion. Some prefer to hold the top plate vertically and resting on a table, then with the razor in the right hand, to use a chopping stroke and cut away from the body, toward the table, cutting downward with a slicing stroke. Subsequent treatment of

sections is the same as for the freehand method, and they are cut wet.

A better system is to imbed the object in the well by using an approach to the full paraffin method of advanced machine microtomes. A mixture of three parts melted paraffin and one of vaseline, thoroughly heated and mixed together at a low temperature is prepared. The well of the microtome is then warmed and filled with this mass, the tissue is dried as much as possible in a cloth, then put into the well and held below the surface with a toothpick or needle dipped into xylene. When the paraffin mass has cooled and solidified just enough to hold the tissue in place, twist the needle, remove it, and plunge the well into a vessel of cold water in order to chill the paraffin quickly. When cold and dried off, the sections are ready to cut. They are cut dry and processed further as by the paraffin technique described in Chapter 14.

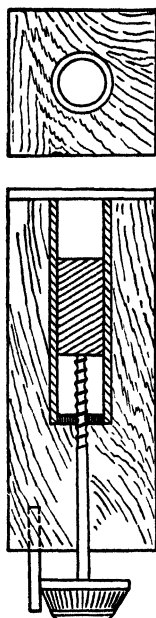


FIG. 107.—Top and sectional views of home-made well microtome. The knob at bottom is a graduated radio dial.

### MACHINE MICROTOMES

Microtome is from the Greek, *small* and *to cut*, and is hence an instrument designed to cut very thin slices. The butcher's automatic meat-slicing machine in your neighborhood meat market employs the same principles as a rotary microtome on a larger scale. In various types of these appliances, there are two important parts, the knife and the object carrier, either of which may be stationary while the other advances a given distance with each revolution. In some the object is held in one place while the knife advances; in others the knife is stationary and the object advances. Either method accomplishes the same result.

There are a great many makes, styles, and sizes of

microtomes, falling within three classifications: freezing, celloidin, and paraffin microtomes; there are also combinations that use two or all three of these methods with a single instrument. Some of the simpler and older models are of

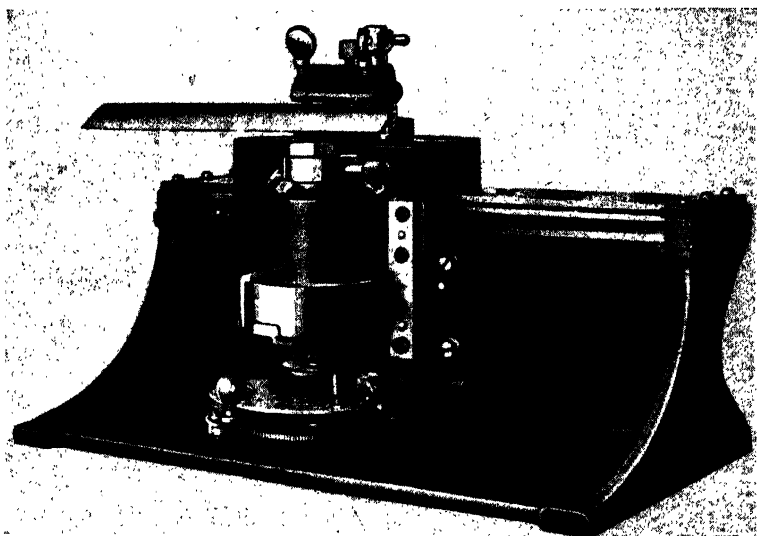


FIG. 108.—Spencer sliding microtome.

the so-called “sledge” type, in which the object is immovable and the knife is carried by a heavy wedge sliding back and forth in a V-shaped trough. The knife is pulled forward by hand, makes a cut, and is returned; then a micrometer screw is turned through a given arc to raise the tissue the desired amount and again the knife is pulled forward for another cut.

The modern descendant of the sledge form is the sliding microtome (Fig. 108), obtainable in various models and capable of use in all three methods though designed primarily for work with celloidin. This style of instrument is essential for sectioning very hard substances and will cut bone, hardwoods, and even metal. Simpler types have a hand feed, others are automatic.

Certain of the smaller freezing microtomes are not much

more than hand microtomes, the cutting implement being a carpenter's plane which is operated on two glass runways, the feed being worked by hand-turning a milled head or wheel (Fig. 109). Improved models are automatic and

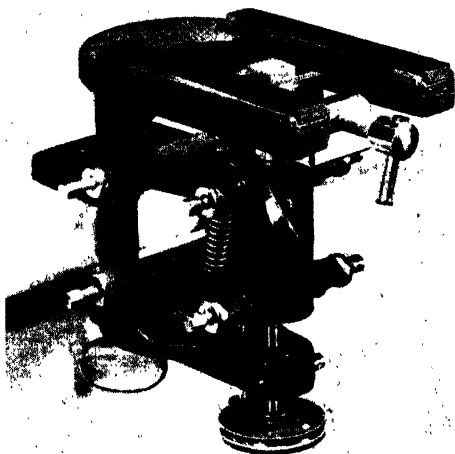


FIG. 109.—Simplest type of freezing microtome. (*Spencer Lens Company.*)

commonly incorporate features to cut sections by all three methods, when they are generally termed "clinical" microtomes (Fig. 110) and designed principally for rapid use in hospitals. A type now obsolete is the rocking microtome; sometimes one of these can be picked up for a small sum and put to very good use.

By far the preferred instrument today for professional sectioning is the Minot automatic rotary paraffin microtome, usually termed simply a "rotary" microtome (Fig. 111). A heavy but finely balanced flywheel in a vertical plane at one side operates the feeding mechanism, which is in the form of a micrometer screw turned by a large ratchet wheel engaging a steel pawl. By setting a cam, one automatically regulates the number of cogs on the ratchet wheel that will slip by the pawl before the latter engages, and hence determines the amount of forward motion im-

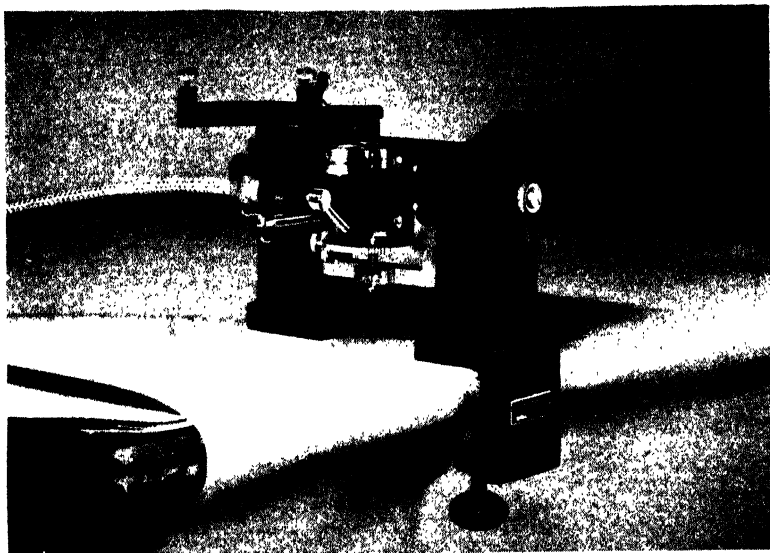


FIG. 110.—Bausch & Lomb simplified clinical microtome, with carbon dioxide freezing attachment.

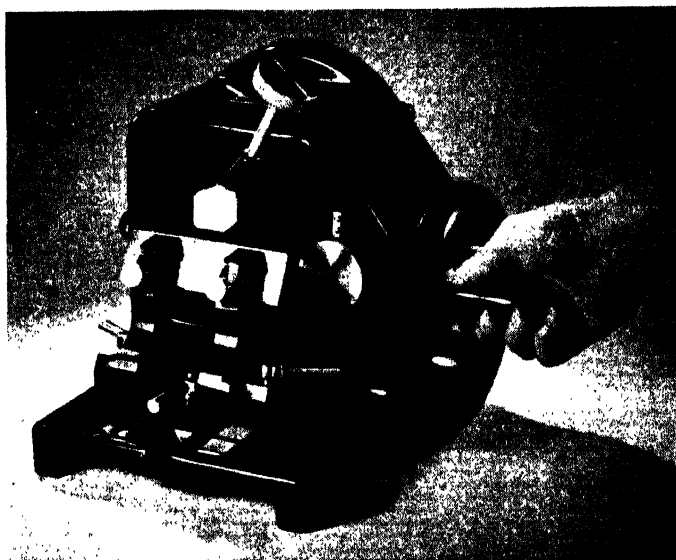


FIG. 111.—Automatic rotary paraffin microtome. (Bausch & Lomb.)



parted to the feeding screw for each cut. Sections from 1 to 25 microns may be cut. This type of machine has the added advantage of cutting serial sections with ease, that is, one section after another of the same object so that when a whole series of them are mounted on slides, one following another, an idea of the three-dimensional construction of the object may be built up.

### FREEZING METHOD

**Description.**—The imbedding medium in this technique is a mixture of gum and sugar; a saturated solution of loaf sugar in distilled water, 30 cc., and gum mucilage, 50 cc. The latter consists of 60 g. of gum acacia dissolved in 80 cc. of distilled water. The principle is to surround and imbed the object to be cut in this mass, which does not crystallize upon freezing and hence will not damage the tissue. Freezing may be accomplished in a variety of ways. The professional laboratory uses a tank of carbon dioxide, such as is supplied to soda fountains, exchanging empty cylinders for charged ones as needed. Occasionally an amateur microscopist has access to such a tank or is able to buy a small tube similarly charged from an automobile supply store. On the whole, however, most amateurs prefer to have recourse to some other method of freezing. Formerly ether or rhigolene was used, with special attachments for freezing; today two other substances are available which are much easier and more satisfactory to work with.

The first of these is ethyl chloride, obtainable from druggists in 50- or 100-g. glass tubes fitted with a release valve. Evaporation is so extremely rapid when the nozzle is opened that the heat of any object touched is extracted with great speed and the object frozen; hence it is important to use such a tube cautiously and not allow any of the substance to come in contact with the flesh.

The second is frozen carbon dioxide, known commercially as dry ice and secured from ice-cream plants. This material is so extremely cold that again caution must be exercised;

handle with forceps only, never with the fingers. It may cause severe burns if it comes in contact with the skin.

**Procedure.**—The following sequences of steps are possible in the freezing method:

1. Fresh tissue is frozen and cut at once, without any imbedding or fixing.
  - a. Such sections may be mounted at once, without any staining or clearing, for immediate examination in glycerin.
  - b. Sections may be stained and mounted in glycerin for temporary examination.
  - c. Sections may be fixed after cutting and then processed through the regular technique for permanent stained balsam mounts.
2. Tissues may be fixed and then cut at once, after which they may be treated as by *a*, *b*, or *c*, above.
3. Tissues may be fixed and washed, then sectioned, after which the regular technique follows to a finished permanent slide.

With either 1, 2, or 3, it is better to use the gum-sugar imbedding mass, though sections will cut without it. Ice crystals will form in the tissues and distort them, however, unless this mass is used.

Choice of these methods will depend on the needs of the case. Where speed is essential, as frequently occurs in hospital diagnoses, 1 or 2 is usually selected; for the amateur, working in his laboratory without the necessity for speedy completion, 3 is greatly to be preferred.

Any of the regular fixing agents may be used, such as Zenker's or Bouin's, but perhaps the best general fixer for this method is 10% formalin. Fix for overnight or 24 hr., then wash in running water for an equal period (Zenker's), or in 50A (Bouin's), or omit washing (formalin). If previously fixed and stored materials are to be sectioned, they should be run down through the alcohols to water and washed well for 12 hr. before being frozen.

After washing, place the tissue in the gum-sugar mass for 24 hr. for infiltration, then remove the tissue and blot off excess sirup with a cloth. Place a small amount of the gum mucilage (*not* the gum-sugar) on the freezing plate of the microtome and orient the tissue in this so that sections will be cut in the desired plane. Now pour some gum mucilage over the object and begin the freezing. If no imbedding mass is used, freeze a little water on the microtome disk in order to contact and connect the specimen.

If the carbon dioxide tank is used, connect the freezing attachment to the tank and microtome, then open the valve from the tank while that to the microtome is closed. Now open the valve at the microtome cautiously and freeze the tissue. It is best to open and shut the valve rapidly several times, as this freezes quickly and saves the gas. If ethyl chloride is used, open the nozzle on the tube and direct a stream against the tissue and plate until the tissue is frozen, as evidenced by its white appearance. If dry ice is employed, first place the tissue on a small block of this material and leave it until frozen; then transfer to a little water on the microtome plate and cap the tissue with the piece of dry ice for a few minutes more.

Sections are cut with the blade of a carpenter's plane, which should be well sharpened beforehand and can be operated more easily if it is mounted in a short, broad tool handle of wood, similar to that supplied as a special instrument by microtome manufacturers. The bevel edge of the plane rests flat on the glass runways of the microtome, in this way elevating the handle toward the operator at about a 45-deg. angle. The handle is held in tightly against the body, supported with both hands, and sections are planed off by bending the body forward at the waist and forcing the plane squarely through the tissue, not obliquely as with the sliding cut of freehand sectioning. The knife must be kept as cold as possible, most easily accomplished by dipping it into a tray of ice water between cuts. Sections must be cut as rapidly as possible, shaving them off in

quick succession; if the feed is by hand, it is best to have a helper work the feeding mechanism for you.

**Difficulties.**—1. The sections crack and are too brittle. Causes; tissue frozen too hard or not enough sugar in the imbedding mass. Correction; let the mass thaw a little and try again. If there is still trouble, reimbed in a mixture containing a greater proportion of sugar.

2. The sections curl or roll. Cause and correction same as for 1.
3. The sections fly off the knife. Cause and correction same as for 1.
4. Sections freeze to the knife and are removed with difficulty. Cause; knife not cold enough. Correction; chill the knife more thoroughly.
5. The sections are crushed instead of being cut cleanly. Cause; the mass has thawed too much and is not sufficiently frozen. Correction; freeze further before cutting again.
6. The whole tissue mass is knocked off of the freezing plate of the microtome. Causes; the tissue was insecurely fastened to the plate or the whole mass has thawed too much. Correction; refasten and freeze well.

**Aftertreatment.**—As fast as sections are cut, they are dumped into a vessel of distilled water, or removed with a brush, poking them gently into the water. It is perfectly all right to allow a number of sections to accumulate on the knife before removing them. Change the water several times to dissolve out the gum completely, then proceed with any desired staining and mounting steps.

**Comparative Merits.**—Each of the three machine methods of sectioning has certain advantages and disadvantages:

*Advantages:* 1. Fresh tissues may be cut by the freezing method without the lengthy procedure of fixing, hardening, and staining, thus very greatly shortening the time con-

sumed before an examination can be made. Any of these more exact processes can be employed if desired, however.

2. Certain objects with parts varying greatly in density, such as a cross section of the finger to include the fingernail, can be cut successfully by this method, and either not at all or very poorly by any other.

3. Material that would be injured by strong alcohol can be cut by the freezing method and mounted in glycerin jelly. This is notably the case with fatty tissue where it is desired to retain and stain the fat cells. Alcohol dissolves out the fat and so cannot be used in this instance. In microchemical work, this method is indispensable.

*Disadvantages:* 1. It is claimed in general that the freezing method is a rough-and-ready one, unsuited to cases where speed is not a factor and where delicate and precise work is required. Lee, on the other hand, describes a more detailed procedure and insists that this technique is entirely suited to the finest cytological work.

2. Tissues do not take stains well unless they have been thoroughly fixed and washed, which does away to some extent with one of the chief advantages of this method—the rapidity with which sections may be made.

3. The method is not suited for making serial sections.

## CHAPTER 13

### SECTIONING: CELLOIDIN METHOD

*In This Chapter:* details of the celloidin and combination celloidin-paraffin methods.

**T**HE imbedding medium in this technique is one of several patented preparations of trinitrocellulose, also termed "pyroxylin" or "soluble guncotton." Celloidin, parlodion, collodion, and photoxylin are the chief commercial preparations of this medium. Granulated, shredded, or tablet forms of celloidin are all available and equally serviceable and are prepared for use by dissolving in ether-alcohol, which is a mixture of equal parts of absolute alcohol and sulphuric ether. (*Caution:* Always be careful to keep all flames away from ether.)

**Procedure.**—In general, the following steps are involved: soaking the completely dehydrated objects in the solvent for celloidin; infiltration with celloidin of increasing strength; imbedding in concentrated celloidin; hardening the celloidin mass to a consistency suitable for cutting. Tissues are fixed, washed, and dehydrated as usual, ending in absolute alcohol for a duration of not less than 1 hr. They are then placed in ether-alcohol for 2 hr., thin celloidin for 24 hr. to several days or even weeks; and thick celloidin for a like period.

Technicians differ greatly in their opinions concerning celloidin infiltration. The most rapid method advocated (Gilson) is to place the tissue in thin celloidin (by which is meant a 2% solution of dry celloidin in ether-alcohol) in a test tube and dip the latter into melted paraffin. This boils down the celloidin, making it more and more concentrated, and continues until the celloidin is reduced to about

one-third its original volume. Some regard one thin (2%) and one thick (8%) celloidin as sufficient; others advocate more gradual steps, as 2, 4, 6, and 8% celloidins.

The method of Eycleshymer is a good one: put a tablet or quantity of celloidin into a wide-mouthed bottle or flask and add enough ether-alcohol to cover the solid. Shake and add more ether-alcohol from time to time until the celloidin is all dissolved; this may take several days. The amount of each ingredient used should be such that the final product has the consistency of a very thick oil. This is labeled solution 4. Mix 2 parts of this with one part of ether-alcohol and label as solution 3. Take 2 parts of solution 3 and 1 part of ether-alcohol and label as solution 2. Solution 1 is the plain solvent, ether-alcohol, with no celloidin. After the object to be sectioned has been completely dehydrated, it is placed successively in solutions 1, 2, 3, and 4. For a piece of tissue 2 mm. square, 24 hr. in each is sufficient; for one the size of a cat's brain, 1 wk. each; for a large brain or embryo, 1 mo. each. It is better to err by leaving too long, rather than not long enough, as prolonged infiltrations do no harm.

For imbedding, one customary procedure is to use a small block of wood, fiber, or vulcanite (Fig. 112), which is later to be clamped into the microtome and used as the object carrier. Grease one surface of a piece of stiff paper with a very light film of vaseline and wrap this around the block, greased face inside, in such a way as to project beyond as a collar in which the object is to be placed. Tie the paper in place securely with thread and pour in some thick celloidin. Pick up the tissue with forceps and put it in the cup thus made; then fill the remainder of the space with more thick celloidin. Dip one or two needles into ether-alcohol and with these arrange the tissue so that it can be cut in the desired plane. As soon as a film has formed over the exposed celloidin, place the block in a small stender dish of chloroform and put on the cover. About 3 mm. of chloroform is recommended and the block does not have to be completely

immersed. From 1 or 2 hr. to as many days will suffice to harden the celloidin which, when ready to cut, should be so hard that it will not be squeezed out of shape when pressed with the fingers nor show a fingerprint, yet can be dented

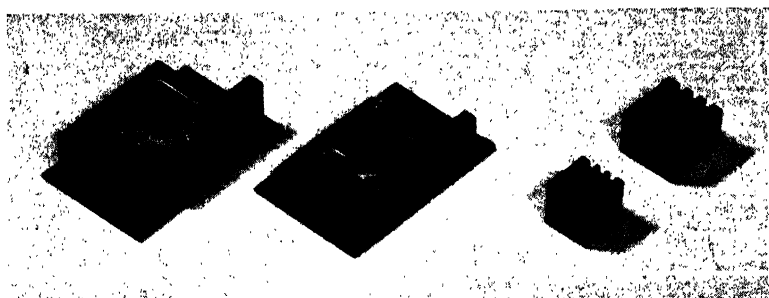


FIG. 112.—Metal imbedding L's and vulcanite object carriers. (*Bausch & Lomb.*)

with the fingernail. When sufficiently hard, the block may be cut at once, or stored until a more convenient time in a jar containing a mixture of equal parts of 95A and glycerin, or in 70A alone.

A second method of imbedding is to construct a paper boat (Fig. 113) especially desirable with large objects, but applicable to all. Select a rectangle of stiff paper and fold it lengthwise twice to divide into thirds. Fold up each end for a depth slightly greater than that of the two sides. When all four folds are made at once, there result four tab ends at the corners, which are turned toward each other at the two ends and the extra length thus left at the top is crimped down over the ends and holds them in place, as shown by the illustration. The finished boat should be large enough to hold the object with a bit of room to spare all around; a little practice is needed to know in advance approximately the right size of paper rectangle to start with in order to make a boat of any desired dimensions. The paper should be lightly greased on the inside with vaseline, and the name of the tissue should be written on the bottom outside with a soft pencil.

Some thick celloidin is poured into the boat to a small



depth and hardened by exposure to the air for a few minutes; then the object is transferred to the boat and more thick celloidin poured in to fill it. The subject is arranged with needles moistened in ether-alcohol and, when a film

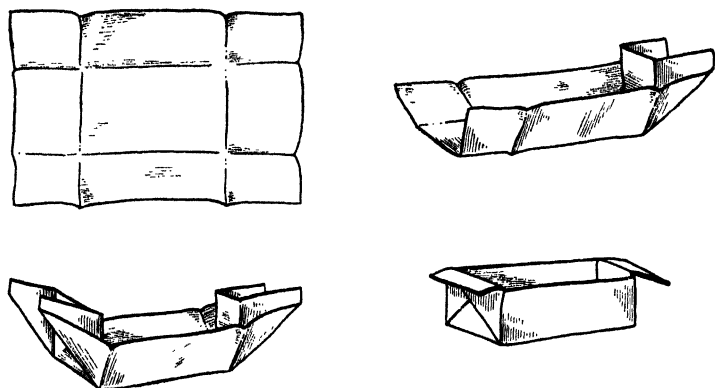


FIG. 113.—Steps in the making of a paper imbedding boat.

has formed over the top surface, the boat is placed in a covered stender of chloroform. Hardening takes place from the vapor of this chloroform as well as from the liquid itself penetrating from the bottom and lower sides. After two days or more, the boat is transferred to the alcohol-glycerin mixture or 70A until you wish to make the sections.

A third method, applicable particularly where a number of separate objects are to be imbedded at the same time, is to pour a little thick celloidin into a glass dish; then add the specimens and pour on more thick celloidin. Orient the specimens at least approximately. Cover the dish loosely and place under a bell jar or inverted finger bowl so that the celloidin can evaporate slowly during one or two days. Harden with chloroform. When ready to section, each object is then cut out from the mass in a block of suitable size.

Metal L's (Fig. 112) may be purchased from dealers and are commonly used to shape the imbedding mass and hold it so until hard.

Regardless of the imbedding and hardening method

selected, the next step is to trim and mount the celloidin block for sectioning. The block containing the object is trimmed down with a knife or razor so that but a small amount of celloidin remains surrounding the tissue. Final correction of orientation can also be effected by suitable trimming.

If not already mounted by the first of the foregoing imbedding methods, the base of the block is now dipped for a few minutes into ether-alcohol to soften it, then into thick celloidin, and the end of the wooden, fiber, or vulcanite object carrier (Fig. 112) is also dipped first into ether-alcohol and then thick celloidin. The two dipped blocks are then pressed tightly together and placed in a dish of chloroform or 82A to harden.

Large celloidin blocks may be clamped directly into the microtome (Fig. 108) without mounting on any object carrier. Otherwise the carrier block is now fastened tightly in the clamp of the microtome and set at the proper level to cut the desired plane of sections. Set the knife at an oblique angle so that it will pass through the object with a long slicing cut. Oblong objects should be oriented with the long axis parallel to the razor blade. Sections are cut wet, both the knife and the celloidin mass being kept flooded with 70A, for which many laboratories rig up overhanging drop bottles. Pull the knife carrier forward with a straight steady pull that avoids bearing down or lifting up; then push the knife carrier back to the starting position. If the feed is automatic, make another cut; if not, raise the object by the feed screw after the knife is back at the start.

The celloidin method works best for thick sections, though with small and fairly soft objects, well infiltrated, and with a very sharp razor, sections as thin as 5 microns can be cut. Ordinarily they are 10 microns or more, 15 or 20 being preferred in general for many types of tissue. Sections are cut rather slowly as contrasted with other methods; each section has to be removed after it is cut, and placed in a dish of 70A. Remove with a forceps (large thick sec-

tions), a strip of stiff paper or cardboard, or a brush. Some technicians draw the sections up on to the back of the knife with a brush and leave them there until a number have been cut, as they do not interfere with sectioning in that location. Do not cut too slowly; the block may dry out to a point where sectioning is not so good.

From this point on, sections may be handled as loose objects in stender dishes, and staining, dehydrating, clearing, etc., performed before they are mounted on slides at all. However, some technicians mount sections immediately after cutting, and one of the time-honored ways is to transfer directly from the microtome knife onto slides, one or more sections per slide, as preferred. To do so, first prepare some albumenized slides as follows.

**Affixing Sections to Slides.**—A very small drop of Mayer's albumen fixative is placed in the center of a thoroughly cleaned slide with the point of a toothpick. With a well-cleaned forefinger, proceed to rub this small drop lengthwise of the slide in both directions until finally the whole surface of the slide is covered and a scarcely perceptible film, evenly distributed, remains. There should be no lumps or ridges of the fixative visible. Gelatin fixative is preferred by many over the albumen type.

Sections are transferred to albumenized slides by forceps, spatula, or brush and are gently pressed down to affix them by covering with a strip of thin paper moistened in alcohol. Then add 95A with a pipette, after removing the paper, and a moment later follow with a pipetteful of 100A. This is a solvent of the celloidin, and only enough of it is used to soften the edges of the section and get it to adhere firmly.

Gage recommends a paper transfer from knife direct to slide. One or more sections are on the knife, after cutting. A piece of white tissue paper (toilet paper is excellent) some 3 by 6 cm. in size is laid over the sections on the knife and pressed down slightly so as to contact all parts of the

sections. Take hold of the paper beyond the edge of the knife and gradually pull it down off the knife. If there is sufficient alcohol on the knife, the sections will adhere to the paper and come off with it. Now press these sections down onto the albumenized slide, cover with a second piece of paper, and press gently. Remove the second sheet of paper. Take hold of the tissue paper by one edge and lift it off with a rolling motion from the slide—peel it off; whereupon the sections should adhere to the slide. Then fasten them on with alcohols as suggested in the preceding paragraph.

By either of the foregoing methods, follow up by draining off the absolute alcohol and adding a few drops of ether-alcohol. This should affix the sections firmly. If the celloidin does not melt somewhat, the dehydration was insufficient and more alcohol should be used. Then let the slide stay exposed to the air for a few minutes, and place it in 70A for 30 min., after which it is ready to stain at any desired time.

Serial sections may be handled by this paper-transfer method or by the following plating or sheet method. There are many of these, the one given being from Linstaedt.

A piece of plate glass is coated with a sugar solution made by adding 3 g. of saccharose and 3 g. of dextrin to 100 cc. of distilled water, and then dried. Coat the plate with a 4 % solution of celluloid in acetone. Cut sections are transferred in proper order and orientation to this plate, keeping them moist with 95A and blotted with toilet paper, treated with alcohols and ether-alcohol, as before described to affix and prevent curling; then they are sprayed from an atomizer with a 2 % solution of celluloid in ether-alcohol. When partially dry, the whole plate is immersed in 70A, then in water. This will dissolve the sugar, and the celluloid sheet bearing the sections will float off. This sheet may be cut and mounted as desired, or may be preserved in 80A.

After a celloidin block has been hardened in chloroform, before cutting, some workers place it in a mixture of 1 part castor oil and 3 parts xylene (castor-xylene clarifier) for a

few days, when it should become entirely transparent, the tissue being as if imbedded in glass. Cutting is the same except that the block and knife are kept wet with castor-xylene and, in affixing to the slides, the sections are flooded with ether-alcohol to make them adhere. As soon as the sections on the slides begin to look dull, put them in xylene to remove the castor oil; then through 100A quickly into 95A and thence to hydrating and staining.

Some technicians prefer the dry method for cutting small to medium-sized objects, preparation for which proceeds as usual through dehydration with 95A. Then put the objects in equal parts of 95A and cedar oil, then pure cedar oil, avoiding absolute alcohol. Next put them in a 6% celloidin and incubate at 37° C. for several days in a corked bottle. Imbed in fresh 8% celloidin, harden in the air a few moments, then in chloroform for 3 hr. Then change to equal parts of chloroform and cedar oil for 3 hr. more and finally to pure cedar oil alone for 3 hr., storing in a tightly stoppered bottle or proceeding to cut as soon as desired thereafter. Sections may be cut then in the regulation fashion on a rotary paraffin microtome, as subsequently described. Cut sections are passed through 95A to 82A and thence to 70A for storage or further processing.

**Celloidin-paraffin or Double Imbedding.**—This procedure is sometimes necessary with brittle and delicate objects, notably eggs, in which cavities and materials of different consistencies are present. Frog eggs, for example, are notoriously difficult to section without crushing or crumbling, and are best handled by this combination method. Celloidin imbedding comes first, then the paraffin; because of the fact that paraffin is to be used, the dehydration must be very thorough.

A more exact though complicated and tedious procedure is that of Apathy, who dehydrates fixed tissue to absolute alcohol, three changes of which are used to ensure complete dehydration. Then the object is passed through ether-al-

cohol for 5 hr., 2% celloidin for 24 hr., 4% celloidin for 24 hr., then imbedded in fresh 4% celloidin and hardened in chloroform vapor for 12 hr. Quickly trim the block to leave a small margin on all sides of the object and immerse in fluid chloroform for 12 hr. Next the block goes into a mixture of 4 parts of chloroform, 4 of origanum oil, 4 of cedar oil, 1 of absolute alcohol, and 1 of carbolic acid crystals, all by weight and not by volume. Some anhydrous sodium sulphate is to be kept in the vessel to take up any water brought over in the celloidin. Leave the block in this mixture until it clears and sinks, which may take several days to a week or more. Next wash in several changes of benzene to remove the oils and alcohol; then infiltrate in paraffin, imbed, and section as explained under the paraffin method. Remembering that absolute alcohol is a solvent of celloidin, observe the precaution, in later staining and mounting techniques, not to leave the section in 100A for any but the briefest periods.

**Stains Following Celloidin.**—The further technique by this sectioning method requires a few special remarks at this point. Generally speaking, most workers make no attempt to remove the celloidin imbedding mass incorporated with each section affixed to a slide, but stain this mass along with the tissues. This usually does no harm, but it is necessary to note that stains take more rapidly under these conditions and should be used diluted. Lee, for example recommends only 0.25 cc. of Erlich's hematoxylin in 50 cc. of distilled water, to be used for several hours or overnight. Most anilin dyes stain the celloidin matrix intensely and are therefore avoided, though Guyer uses safranin or eosin and removes these dyes from the celloidin with acid alcohol before too much has been extracted from the tissues.

When anilin dyes are to be used extensively—and some workers prefer this method for all cases—the celloidin may be removed from the tissues before staining, provided they will not disintegrate when this support has been dissolved

out. To do so, pass the sections through absolute alcohol into ether-alcohol, then back through absolute into the lower alcohols.

**Difficulties.**—Nearly all of the difficulties likely to be encountered in using the celloidin method can be traced to insufficiencies in dehydration, infiltration, imbedding, or hardening. All these steps must be very thorough and slow, and it is always better to leave the blocks in the various reagents for a longer rather than a shorter time. One cannot cut corners in this method.

**Comparative Merits.** *Advantages:* 1. The celloidin method employs no heat, as does the paraffin; consequently, objects that might be injured by heat are better handled in this way.

2. Large objects may be cut more smoothly, without distortion or crushing.
3. Brittle objects cut better than by the paraffin method.
4. Certain tissues are not rendered so hard or so difficult to section as by the paraffin method.
5. Objects with cavities and loose membranes or internal parts are held together better.
6. There is less shrinkage and distortion of material.
7. Hard objects, as chitin, nail, horn, tendon, and fibrocartilage, cut much better and more naturally.
8. A greater affinity for stains is present in celloidin-imbedded tissues.
9. Botanists are returning more and more to the celloidin method for the most perfect sections of hard parts, especially stems and roots.

*Disadvantages:* 1. The method is extremely slow, the slowest of all.

2. By the usual wet method, the work is messy and not so pleasant as in paraffin sectioning.
3. Except by experts, thin sections cannot readily be cut. Mostly they are 15 to 20 microns.

4. Serial sections are not easily secured. They are possible but only after considerable labor and practice.
5. The celloidin mass is stained as well as the tissue and not all workers advocate its removal. Some delicate parts may be lost thereby.
6. Not so many stains may follow this technique as in paraffin sectioning.



## CHAPTER 14

### SECTIONING: PARAFFIN METHOD

*In This Chapter:* various procedures and imbedding media used with the paraffin method; summary of sectioning methods; sample technique schedules for vertebrate histology by the three machine methods.

**I**N THE ordinary routine by this method, the material used is paraffin and the principle is to permeate the tissues thoroughly with a mass that is fluid when heated and solid when cooled to room temperatures. Paraffin meets these conditions since it is rendered liquid at relatively low temperatures that will not ordinarily harm the objects to be sectioned.

Pure paraffin especially packaged for sectioning is available from all supply houses and is put up in blocks of various degrees of hardness, indicated by the melting point. One catalog, for example, lists four grades, with the following m. p. (melting point): 47 to 49; 50 to 52; 53 to 55; 56 to 58, all in centigrade, corresponding to a range of 118 to 135° F. For exact work or special temperature conditions, as in very cold or very hot weather, it would be better to use such paraffins; for all ordinary work under average conditions, the much less expensive parawax or other household preparation for sealing preserves is entirely satisfactory.

A low-temperature oven of some sort is essential in the infiltration and imbedding, in order to keep the paraffin melted at the desired degree, and it is quite important that the heat should not rise much above the melting point of the paraffin used, in order to avoid cooking and ruining the tissues. In previous chapters references have been made to commercial or homemade slide driers or ovens and incubators for bacteriology; any of these may also be used for paraffin imbedding. Still other simple expedients have been

widely recommended by many authors. For example, a beaker of paraffin placed on the table may have directly over it a student lamp so that the shade almost touches the beaker, leaving a small air space. Heat from the electric bulb will melt the paraffin down to a certain level only, not all the way, and the object being infiltrated will sink to that level and remain there at a point where the paraffin is just barely melted—an ideal condition. An electric hot plate or section spreading board may be used after determining that point on the sheet of metal where the paraffin will just stay melted.

**Procedure.**—Material must be thoroughly dehydrated, since paraffin is not miscible with water. The shortest method in general use, and one well suited for routine work, is to pass the object up through the alcohols, after fixation and washing, 20 to 30 min. each, into absolute alcohol for 30 min., absolute and xylene, half and half, for 15 min., xylene for 1 to 2 hr.; or, from 95A to carbolxylene, then pure xylene. Next, xylene and melted paraffin, half and half, for 30 min. This mixture requires only a slight rise in temperature to stay melted. A stender or beaker containing material in this reagent is commonly placed on the top, outside, of an oven or incubator—any situation of very low heat is satisfactory.

A bath in melted paraffin next follows over a period of from 1 to 3 hr., and best results are obtained by transferring the object twice to fresh paraffin in order to get rid of all traces of the xylene. In practice, three stenders are prepared in advance, labeled paraffin 1, paraffin 2, paraffin 3. Each is filled with paraffin, covered, and placed in the incubator. In order to ensure thorough infiltration, it is well not to have the object resting on the bottom; the customary device to prevent this is to sling a narrow strip of paper across the dish, sagging in the middle to about half of the depth, and with the two ends crimped over the sides as support. The object is transferred by forceps or spatula

from one dish to the next, being placed upon the paper sling in each case.

The length of time in each bath will vary with the object to be sectioned, but should never be longer than necessary. Large, hard, solid, and impervious subjects require longer times than do small, soft, hollow, or easily pervious ones. Fifteen minutes in each bath is a minimum, 1 hr. in each is sufficient in most cases, and 2 to 3 hr. in each is a maximum except in extraordinary cases.

When ready to imbed, make a paper boat (Fig. 113) or use metal L's (Fig. 112) of the proper dimensions and pour in some fresh paraffin to a depth of about one-third the container. Allow this to harden just a trifle, not enough so that, when the box is filled, there will be a line of cleavage between the first and second increments of paraffin. Warm a forceps and transfer the object quickly, so that no film of hardened paraffin forms on the exterior. Remember that paraffin hardens rapidly after removal from the source of heat. Fill the receptacle with more fresh paraffin, then with a pair of warmed needles, orient the object with its long axis coinciding with that of the imbedding box, and straight so that true cross sections may be cut. Support the object until the solidification of the paraffin is sufficient to do that for you; then quickly twist (twirl) the needles to free them and remove them. As soon as a thin surface film has formed over the paraffin across the exposed surface, plunge the box into a beaker of cold water into which a tap is delivering a stream (running water). Some workers advocate ice water, others prefer to use alcohol. The idea is to congeal the paraffin quickly to prevent crystallization.

Remove the paper and examine the block when cold. If there is a sharp cleavage line between the top and bottom layers, or if there are many and large white opaque spots or areas present, particularly near the object, it will be necessary to reimbed. The opaque spots are caused by alcohol or xylene carrying over into the paraffin and will interfere with sectioning. Melt the paraffin and repeat the

infiltrating baths; then reimbed. If the top surface of the paraffin has collapsed upon cooling, forming a depressed area, no harm results unless the object is insufficiently covered.

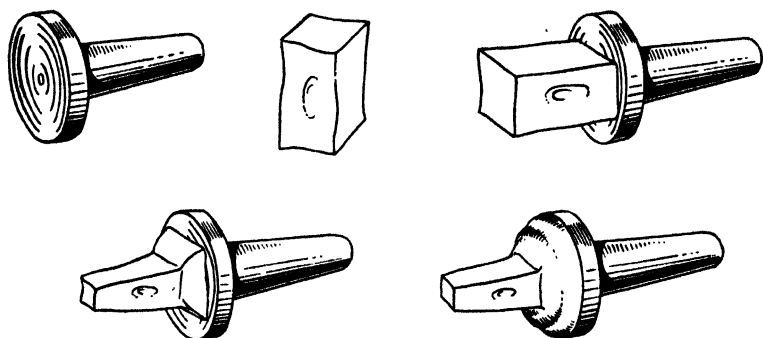


FIG. 114.—Steps in affixing paraffin block to object carrier, trimming block, and building up paraffin shoulder.

Paraffin blocks may be left indefinitely before sectioning. Many laboratories have collections of tissues in this form which they may keep for years before using; the material is permanently protected in this form. Always write the data on the outside bottom on the paper, however; do not trust to the memory to identify a particular block.

When ready to section, remove the paper and trim the block by chipping off the paraffin in very thin slices with a knife, but do not carry the lengthwise cuts all the way through; leave the original mass of the block at the butt where it is proposed to fasten the block to the object carrier of the microtome (Fig. 114). Some prefer to do much of this trimming before the block is affixed to the carrier; others, after such fastening. Some trim down very close to the tissue, so that little paraffin is left; others like a wider margin. About 2 mm. of paraffin all around the tissue is advised. Fasten the base to the carrier by first warming the face of the carrier, then melting a few chips of paraffin upon it, then warming the butt of the block, and pressing the two quickly and tightly together, holding them thus a moment,

and plunging into a beaker of cold water to set and harden (Fig. 114).

Now a shoulder is built up around this connection, and a section lifter or other metal spatula is the preferred instrument. Pick up a chip of paraffin on the spatula, warm it over an alcohol lamp, apply it to the connection between the block and the carrier, and smooth with the spatula just as a mason smooths mortar with a trowel. Continue until a considerable sloping shoulder is built up all around, from the base of the tissue-bearing portion of the block, sloping down to the surface of the carrier (Fig. 114). When finished, chill in cold water, then wipe dry and mount in the microtome clamp. It will be observed that this clamp is provided with lock nuts that permit rotation in all three planes. Set the carrier so that the face of the block to meet the knife is as square as possible, both vertically and horizontally, as judged by careful inspection by the eye, the long axis of the face of the block parallel to the knife blade. Then tighten all clamps until they are very rigid.

Adjust the blade in the knife carrier so that it has a decided tilt inward, toward the paraffin block, about 10 deg. from the vertical, and tighten the knife tilting clamps securely. Cautiously turn the rotary wheel by hand (Fig. 111) to start the object down toward the knife, sliding the knife carrier toward the object until it is seen by close inspection that the further lowering of the object will just barely miss the knife. Then tighten the knife carrier clamps. All clamps should be very rigid when ready to cut.

Set the cam to cut sections of the desired thickness; 10 microns is standard for routine work and the best to use until one becomes thoroughly familiar with microtome operation. Now turn the operating handle on the wheel; it will probably be necessary to make a number of revolutions before the knife contacts the paraffin block, and a number more before one gets down to the level of the tissue. While reeling off merely paraffin, however, is the time to see that the block is cutting properly. Each rectangle of paraffin—

let us call them sections, even though empty of tissue as yet—should cling to the knife after being cut, and at the next revolution should become affixed to the next section, and so on, forming a paraffin ribbon, a whole string of sec-



FIG. 115.—Cutting a ribbon of sections with a paraffin microtome. (*Spencer Lens Company.*)

tions, all affixed end to end to each other (Fig. 115). This is one of the greatest advantages of the paraffin method and unless it is working properly, something is the matter and adjustments should be made before the tissue is reached.

Sections are cut rapidly, sometimes almost as fast as the crank can be turned, only stopping if there is trouble. Pick up the ribbon with a camel's-hair brush held in the left hand and lift it up and away as the ribbon lengthens. With a bit of practice, when cutting conditions are good, a ribbon 1 ft. long may be thus supported before it breaks or the

operator decides to pause in order to take care of it. Ribbons are laid down carefully on a sheet of glazed paper on the table, and nothing should touch them. Be careful of dust and do not breathe or cough on them as they are exceedingly light and will blow away; beware of drafts. Never touch them with the fingers as the heat of the body will melt the paraffin and cause that portion to adhere to the finger. When the ribbon breaks or is purposely broken, continue the support with the brush in the left hand, and use another brush, a needle, toothpick, glass rod, or other implement to get underneath and support the right-hand end of the ribbon; carry it gently across by means of these two supports and lay it down on the paper, straightening it out as needed. Then proceed to cut the next portion of ribbon.

These paraffin ribbons may be stored if desired, but they are so easily blown, melted, stuck together, or otherwise damaged that storage is risky. It is much better to mount the sections on slides right away. One or more individual sections, as preferred, are cut from the ribbon with a sharp knife or safety-razor blade and carried by a section lifter or brush to an albumenized slide that has been flooded with distilled water so that the whole surface is covered—but not enough water so that it will run off the slide. The slide is now placed on a slide warmer for flattening the sections; in England they call it “stretching” the sections. Many types of appliance are available for this purpose, the only requirement being a gentle heat that will warm without melting the paraffin. If, for example, 52° paraffin was being used in the imbedding, any warm temperature 50° or less would answer.

One of the oldest forms of warmer was a triangle of copper or brass mounted on three legs so that an alcohol lamp could be placed beneath the base of the triangle. At some point between this base and the tip would be found an area of the right temperature for the purpose; nearer this base, a beaker or stender of paraffin for infiltration could be placed at the same time, the heat there being

greater. Another form of slide warmer, similar but rectangular and with four legs, is easily made from a piece of brass or copper sheeting, with legs of any preferred metal (Fig. 116).

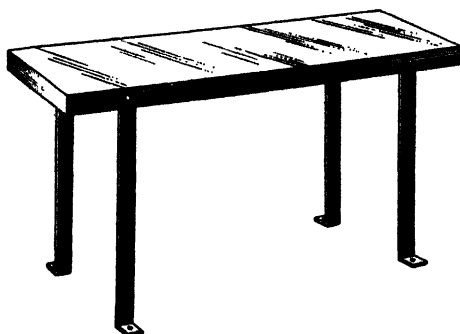


FIG. 116.—Metal slide-warming table.

An electric plate is easily constructed and very serviceable. Secure a brass or copper plate about 12 by 6 by  $\frac{1}{8}$  in., and braze or bolt to a box of any convenient sheet metal, such as galvanized or tin, 10 by 6 in., top outline, the plate forming the lid, but projecting somewhat (1 to 2 in.) beyond the box at one end to give an overhanging cool area. This box may be 6 in. or less in depth, should be lined with sheet asbestos, and the heat supplied by a 40-watt bulb, at one end near the bottom, for which the box is wired. Vessels of paraffin for infiltrating and imbedding may be placed inside or on the top as desired; slides are spread on the top plate. Since the bulb is near one end of the box, all degrees of heat are available on the top plate, which is most useful in many additional operations: drying films of blood and bacteria, drying out cells and rings in turntable mounting, and so on.

It will be found that paraffin ribbons placed on such a warming device will flatten out perfectly, and wrinkles will disappear. Usually the section will stay where it has been placed if the warming table is perfectly level, but as the water evaporates and the paraffin section is drawn down



into the albumen fixative ever more intimately, the time shortly arrives when the position cannot longer be changed; so have the sections oriented in relation to the axis of the slide in the way you wish. If more than one section per

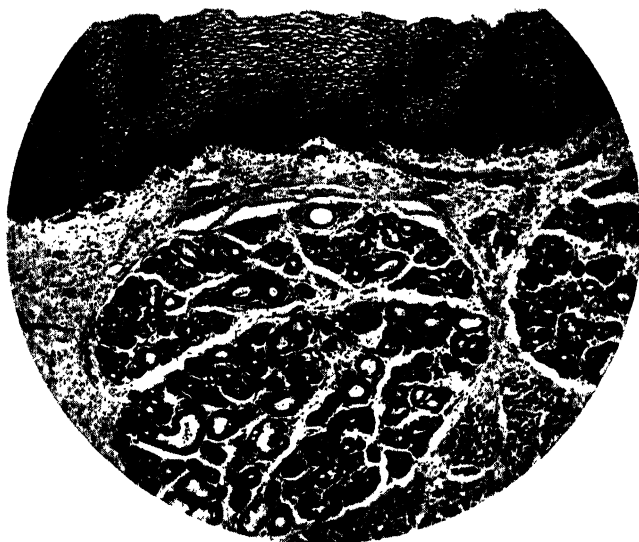


FIG. 117.—Machine-microtome section of human lip, stained, 50 X. At top, cells of the epithelium or surface layer of skin; below, a gland.

slide is affixed, see that they all face the same way and are oriented alike.

If air bubbles form under parts of the paraffin ribbon not involving the tissue, remove them by pricking with a warmed needle; if under the tissue, stroke them away gently with a brush before the slide dries; if present in dry slides, the sections will probably come loose during staining.

When dry, put the slides away in a slide box or dust-free place to continue drying for overnight or 24 hr. before attempting to stain. If protected from dust, such slides may be stored indefinitely, for years if desired, as the tissue is completely surrounded and protected by the film of paraffin.

In staining, the first step is to place the slide in xylene to dissolve the paraffin for 15 min. Warming the slide on a spreader first will hasten the process by partially melting the paraffin. Slides are then hydrated through the alcohol



FIG. 118.—Machine-microtome section of portion of human neck, stained, 50 X. Cartilage of trachea at bottom; above and at right, portion of aorta; at left, of lymph gland; nerves at top and center.

series to water and then stained. Photomicrographs of two finished slides are shown in Figs. 117 and 118.

**Alternative Methods.** 1. *Toto Staining:* Many workers prefer to impart more or less color to the object before imbedding in paraffin, as it may then be seen through the paraffin mass more easily. Borax carmine and alum cochineal are widely used for complete toto staining of the whole object, but this practice has diminished since it is not so

precise as staining individual sections and does not give the contrast afforded by double staining. A very dilute Congo red is excellent in order to tinge the material for orientation and so that sections can be seen more easily in the paraffin after cutting. This stain may be applied as a 0.1% solution in 70A during dehydration before paraffin infiltration.

2. *More Gradual Transitions:* Comparative rapidity is one of the advantages of the paraffin method, yet better results for more critical studies can be obtained if time is not a factor and all the transitions from one type of reagent into another are made gradually. This is especially true in refined cytological studies. The wick or siphoning method (page 163) for increasing the strength of alcohol during dehydration is one example; another suggestion is that of Guyer to add xylene, a little at a time, to the absolute alcohol by using a pipette and running in the clearer at the bottom of the alcohol container. Paraffin may be shaved or chipped into the pure xylene gradually at room temperature. Tobias recommends placing paraffin in a cheesecloth bag and suspending this in the clearer so that it will go into solution gradually, adding more paraffin as may be necessary until the xylene is saturated.

3. *Other Clearers:* Although xylene is perhaps the most generally used of all clearers, it does have certain disadvantages. It is very sensitive to water and will ordinarily clear tissues only from 100A; also it renders many tissues too hard and brittle, being especially bad for eggs rich in yolk. Many technicians prefer cedar oil; in fact they term this the best of all general clearers and almost essential with eggs. It will clear from 95A, though the best procedure is to go through 100A anyway. It does not shrink or render tissues excessively hard and brittle and does not extract anilin dyes. Of the various grades sold, one is designated "for clearing"; another, more expensive, is expressly for use with oil immersion objectives. Material is transferred from 100A to a mixture of equal parts of 100A and cedar

oil for 20 min., then into pure cedar oil for 1 to 2 hr. or until the object looks clear and translucent. One test is that, when clear, the tissue will sink in the oil; if it does not after a while, it was not completely dehydrated. Cedar oil is slower than xylene, but objects may be left in it indefinitely. As a general rule, clear for a time twice that used for xylene, and infiltrate in paraffin one and one-half times as long as if xylene were used.

Oil of bergamot clears quickly from 95A, but has the lowest index of refraction of all the clearing oils. Oil of origanum will also clear from 95A but extracts anilin dyes to some extent. Beechwood creosote is very good to clear celloidin sections and various watery preparations and in clearing for whole mounts. It will clear from 95A; material may be stored in it indefinitely if placed in the dark or in a colored glass bottle. Chloroform has had many adherents as a clearing agent. It is a fine dealcoholizer and will take up excess water; it does not render tissues brittle. On the other hand, it has very poor qualities of penetration and is slow; hence it is used mainly with small objects. Multiply the time of clearing in xylene by 4 or 5 to get the proper length of chloroform clearing; the paraffin bath by 2 or 3.

Benzene (benzol) and toluene (toluol) are similar to xylene, and the use of carbolxylene has already been discussed. Turpentine is generally considered inferior to xylene. Anilin oil is sometimes employed, especially by beginners by way of makeshift, but unless its use is attended by numerous qualifications it is not to be recommended. It will clear from the lower alcohols or even from water and hence has its value for tissues that would be harmed by alcohol; but it oxidizes, absorbs water, discolors, and does not mix well with paraffin, hence its use must be accompanied by an intelligent understanding of its properties. Objects in 50A may have anilin oil added gradually until a transition to the pure oil is attained, keeping all containers covered to exclude air. Then the anilin is washed out with several changes of chloroform before infiltration with paraf-

fin is begun. McClung prefers to pass from anilin oil to oil of bergamot, cassia, or wintergreen to complete the clearing.

**Other Imbedding Media.**—Not every subject will cut well in paraffin, as previously noted, and a combination of the celloidin and paraffin methods for brittle objects, such as eggs, has already been described (page 250). Here are two other useful routines:

1. Bayberry wax, added to paraffin in the ratio of 1 wax to 10 paraffin, increases the hardness of the mixture and lowers the melting point.

2. Johnson's rubber-paraffin method is one of the very best for cutting hard and brittle objects and is recommended by some for all paraffin sectioning. One part of crude India rubber is cut into very small pieces and mixed with 99 parts of hard paraffin which has previously been melted and tinged a light amber color with a little asphalt. Heat the mixture to 100° C. for 24 to 48 hr., or at a lower temperature for a longer period. Pour off the supernatant fluid—the only part to be used—and cool it at once, allowing to remain cold until needed, since it will decompose if kept heated. Use it as you would ordinary paraffin in infiltering and imbedding.

**Difficulties with the Paraffin Method.**—Guyer, in his splendid and well-known manual (*Animal Micrology*, University of Chicago Press), gives a lengthy and very complete list of all of the troubles likely to be encountered in sectioning by the paraffin method, together with the suggested causes and remedies. The following reproduces the more essential points in condensed fashion:

1. Crooked ribbons. *Causes*; (a) Wedge-shaped sections. See that block strikes knife exactly at right angles and have opposite edge of block exactly parallel with this first edge. (b) Unequal sharpness along knife-edge.

2. Object scratches on knife or cuts with a gritty feeling. *Causes*; (a) Overheating in paraffin; object worthless.

(b) Crystals of fixer (*e.g.*, corrosive sublimate) not washed out. (c) Object contains lime; often true of pathological tissues.

3. Sections wrinkle or jam together. *Causes*; (a) Dull knife. (b) Insufficient tilt to knife. (c) Paraffin on knife-edge. (d) Insufficient infiltration in paraffin. (e) Paraffin too soft. *Remedies for (e)*; (1) Cool block in cold water. (2) Cut sections in a colder room. (3) Cut sections thicker. (4) Reimbed in harder paraffin. (5) Employ a cooler, as a tray or pitcher of ice or dry ice, suspended over microtome.

4. Sections roll and refuse to ribbon. *Causes*; (a) Paraffin too hard. *Remedies*; (1) Breathe on the knife. (2) Cut in a warmer room. (3) Place an electric lamp near the microtome. (4) Warm knife *very carefully* by holding the *back* of the knife on a warm plate. (5) Cut sections thinner. (6) Reimbed in softer paraffin. (7) Dip the end of the block in melted softer paraffin. (8) The sections may uncurl when placed on warm water. (9) When a section begins to roll, hold down its edge with a brush and try to get a ribbon started in this way, then cut rapidly; 2 and 5 are the best of these remedies. (b) Tilt of knife too great. (c) Knife dull.

5. Sections split longitudinally or are crossed by parallel scratches. *Causes*; (a) A nick in the knife blade; cut with a new portion of the blade or sharpen the razor, or use a new safety-razor blade. (b) A hole in the paraffin; reimbed. (c) Grit in the paraffin; reimbed, cleaning well in the clearer. (d) Hard substances in the tissue, as unwashed-out crystals of fixer, silica, lime salts; discard and take a fresh piece of tissue. (e) Tilt of knife too great. (f) Paraffin or dirt on edge of knife. (g) Object too large to be cut in paraffin; use smaller pieces or celloidin method. (h) Paraffin layered owing to slowness in adding second layer when imbedding.

6. Knife scrapes or rings in passing back. *Causes*; (a) Tilt too great or too little. (b) Object too tough or hard to cut in paraffin, causing knife-edge to spring. (c) Blade of knife too thin; often true of safety-razor blades; try a sectioning razor or knife.

7. Sections vary in thickness; the machine cuts one thick and one thin or misses a section. *Causes*; (a) Defective machine with worn parts; tighten up all parts. (b) Same as 6(b). (c) Too great or too little tilt. (d) See that all clamps holding object carrier and knife blade are tight.

8. Object crumbles or drops out of the paraffin on sectioning. *Causes*; (a) Insufficient infiltration in paraffin. (b) Clearer not all removed, particularly likely with cedar oil; use xylene to rinse off cedar oil. (c) Object impervious to paraffin; use celloidin or celloidin-paraffin methods. (d) Object very friable, as many ova; use these other methods.

9. The ribbon twists or curls about or clings closely to side of knife. *Cause*; electrification. *Remedies*; (a) Postpone cutting until atmospheric conditions have changed. (b) Boil some water near the microtome so that water vapor is given off into the surrounding air (Gage).

10. The cut section catches on and clings to the block as it returns instead of remaining on the knife. *Causes*; (a) Dull knife. (b) Edge of knife dirty. (c) Tilt insufficient. (d) Paraffin too soft. (e) Room temperature too high. Toughened tissue caused by too high temperatures or too great hardening by reagents is usually responsible.

In general, observe the following precautions: use a harder paraffin in summer, a softer one in winter. Use a harder paraffin for thin sections, a softer one for thick sections. See that dehydration, clearing, and infiltration have been thorough. Keep the knife blade sharp and clean, and have the tilt of the knife correct. See that the paraffin block is trimmed and mounted properly, with top and bottom edges parallel to each other and to the blade. Attention to these requirements will prevent nearly all troubles.

**Comparative Merits. Advantages:** 1. Intermediate in speed; rapid as compared with celloidin method.

2. Cleaner; sections cut dry.

3. Serial sections obtained with ease.

4. Thinner sections than by any other method.
5. Storage in the paraffin block or affixed-to-slide stages indefinitely without risk.
6. Imbedding mass removed; appearance of finished, stained slide hence often superior.
7. A wider selection of stains may follow.

*Disadvantages:* 1. Heat required; injurious to certain delicate objects.

2. Large, hard, tough, and brittle objects do not cut so well as with celloidin.
3. The technique renders some tissues too hard to cut well.
4. Imbedding mass is removed. This may be a disadvantage in very delicate objects where the various parts need such support.
5. Imbedding mass not so transparent; orientation more difficult.
6. Paraffin causes a certain amount of shrinkage while cooling.
7. Usually more or less distortion of the object.

#### SUMMARY OF SECTIONING METHODS

**Freehand.**—For temporary mounts, either stained or unstained, in water or glycerin. Teachers often prepare these sections fresh and use them for classroom instruction, then throw them away. Research workers may test certain objects quickly in this way to see if the material is what they want, and worth processing by one of the lengthier methods. All microscopists should make a few freehand sections to learn the method.

**Well Microtome.**—With some experience, excellent sections may be prepared, especially if the full paraffin infiltrating and imbedding technique is followed, as if for use with a rotary microtome. This is the method recommended for the majority of amateurs, who do not have a fully equipped laboratory with expensive microtomes, and for all beginners.



**Freezing.**—Widely used by hospitals and for clinical work. Much quicker and simpler than other machine methods, but gives cruder results except in the hands of experts.

**Celloidin.**—Very elaborate and lengthy. Must be used for certain cases where other methods give inferior results, but will be avoided by all amateurs save those who go into the subject exhaustively.

**Paraffin.**—The general rule advocated by nearly all technicians is to use this method wherever possible. In the words of Chamberlain, "Use paraffin when you *can*, celloidin when you *must*."

#### SAMPLE SCHEDULES

In order that the student of microtechnique may acquaint himself with the practice of various techniques, some suggested procedures are here appended in outline form. Although few readers will have access to microtomes suitable for all of these exercises, some will have one type and some another; but all will wish to know at least the methods. At the same time, practice in the use of different fixers, dehydrators, and clearers is given, all of which can be adapted to any of the sectioning methods; the same holds for staining techniques.

**General Preliminaries.**—Capture an ownerless alley cat, or purchase one, and kill it with illuminating gas. Avoid possible legal complications through getting someone's pet. A rectangular aquarium with glass or board lid, a small galvanized trash can, or a tightly built wooden box are all suitable as containers for this purpose, the only requirement being a small hole through which a rubber tube or business end of a bunsen burner can be introduced to admit the gas. Be sure to keep all flames away. Turn the gas on for 3 min., then turn it off and leave the cat in the receptacle 3 or

5 min. more. Remove the cat from the container at an open window or outdoors, to dissipate the fumes, and dissect immediately, to obtain tissues while perfectly fresh.

The city pound or the S. P. C. A. may be called upon to supply a specimen of freshly killed dog or cat, without charge. Slaughterhouses are excellent sources, where permission may usually be obtained to take small bits of organs from freshly killed pigs, sheep, and cattle. Take a few bottles of fixing fluids and your dissecting kit along with you, to bring back the tissues. Frogs, snakes, turtles, chickens, and pigeons are other forms of higher animals accessible for this work. For the great majority of tissues and organs there is no particular advantage to be gained by trying to get human specimens; for example, the stomach or pancreas of the cat or dog is fully as good and representative and much easier to obtain in the absolutely fresh condition so essential for good results. However, certain readers may wish to have sections of human organs; there is perhaps a small element of added interest to such slides. The person to contact is a surgeon, medical examiner (the modern form of coroner), hospital interne, or medical student. Certain materials, such as an appendix or tonsil, are frequently available in the fresh—though usually diseased—condition from the operating room; others are more likely to be secured from post-mortems and are generally far from fresh, though often still useful. Skin, for instance, does not have to be removed from the body so quickly after death as does brain or intestine in order to obtain good results.

Suggested structures for fixing and later processing at leisure are the following: Digestive system: tongue, esophagus, stomach, small intestine, large intestine (colon), caecum (pig or rabbit), appendix (man), salivary glands, liver, and pancreas. Respiratory system: trachea, lung. Vascular (circulatory) system: heart, artery, vein, spleen. Excretory (urinary) system: kidney, ureter, bladder. Reproductive (genital) system: gonad (testis or ovary),

uterus (female mammal). Endocrines (ductless glands): thyroid, adrenal. Nervous system: cerebrum, cerebellum, medulla, spinal cord, nerve, nerve endings in muscle. Muscular system: a piece of muscle from the diaphragm or leg. Skeletal system: a bone, as the femur, cartilage of external ear, cartilage of lower half of rib (mammal) or from sternum (frog), tendon. Integumental system: skin, scalp.

The beginner must learn to take *small* portions of these structures. With hollow, tubular organs, such as the intestine or an artery, or solid cylinders like nerve and tendon, cut with sharp scissors squarely across the tube, then again about  $\frac{1}{4}$  in. farther along and use only this small  $\frac{1}{4}$ -in. piece, ample for many sections. Fixers will not penetrate large pieces promptly enough. A piece of the wall of the stomach, bladder, lung, and similar organs about  $\frac{1}{4}$  in. fore and aft by  $\frac{1}{2}$  to  $\frac{3}{8}$  in. in width is sufficient. Be sure to cut straight cross or longitudinal sections; avoid oblique cuts, which are very confusing as finished slides. As each piece is cut, hold it by a forceps and slosh it around for a moment in a vessel of normal saline to remove blood, hair, and other foreign matter; then place it immediately in the fixer, the volume of which should be from ten to one hundred times that of the object. Several objects may be placed in the same fixing jar provided their appearance and nature are such as to enable identification later, when processing them in your own rooms. Otherwise use separate, labeled vials of fixer for each organ.

In the case of plant tissues, the main parts wanted for anatomical studies of the higher plants are root, stem, leaf, flower, and fruit, and both monocots (as corn, lily) and dicots (sunflower, buttercup, basswood) should be represented. Rhizomes and fruiting bodies of mosses and ferns make very fine preparations. Use fully developed but *green* stems and new roots of woody plants, before they become too hard for routine cutting. Obtain small pieces and place them at once in the fixing solution.

**Schedules.—***Freezing Method:*

1. Fix a small piece of liver in 10% formalin for 12 hr. or overnight.
2. Wash in 70A, 24 hr.
3. Pass through 50A, 30 min.
4. 35A, 30 min.
5. Wash in running water, 12 hr. or overnight.
6. Place in gum-sugar solution, 24 hr.
7. Put a small amount of gum mucilage on freezing disk of microtome and freeze it.
8. Wipe the specimen with a cloth to remove excess gum-sugar.
9. Place object on frozen spot on microtome disk, orient, and freeze it.
10. Add more gum mucilage and freeze; continue until tissue is completely surrounded by frozen mass.
11. Section, planing off slices rapidly. Cut sections not less than 15 microns thick, and keep knife cold.
12. Place sections in a dish of distilled water; change water several times.
- 13a. Using syracuse watch glasses or similar small vessels for the various reagents, transfer a section to alum cochineal, 6 to 12 hr. or overnight. It is well to use the first section as a test to determine how long the rest should be stained for best results.
- 13b. An alternative method is to affix sections to slides at once. Transfer a section to a slide with a brush or small glass rod, straighten it out, blot off water gently, treat with 95A, blot, then with 100A, and blot again. Put on a drop or two of thin celloidin, allow to stand for a few minutes, then immerse in 82A for 5 min. Place slide in distilled water, 5 min., then into the stain, as by 13a.
14. Rinse in distilled water.
15. 35A, 3 min.

16. 50A, 3 min.
17. 70A, 3 min., or, if destaining seems desirable, 70% acid alcohol until decolorized sufficiently, then rinse in fresh 70A.
18. 82A, 3 min.
19. Counterstain with Lyons blue in 95A, which acts very rapidly. Try 10 sec. with the first section, rinse in fresh 95A, and inspect under the microscope. Use 20 sec. for a second section and 30 sec. for a third, or stain one section three times in succession for 10 sec. each, thus determining the length of time that gives the best results.
20. 95A, 5 min.
21. Carbolxylene, 5 min.
22. Xylene, 3 min.
23. If 13a was selected, drain the section against the edge of the dish, transfer carefully to the center of a clean slide and mount in balsam. If 13b was used, merely add balsam and the cover glass.

*Celloidin Method:*

1. Fix a piece of tendon in Gilson's fluid, 6 hr.
2. Wash in running water, 6 hr.
3. 35A, 30 min.
4. 50A, 30 min.
5. 70% iodinated alcohol, 24 hr., adding more iodine solution if decolorized.
6. 70A, two to three changes, 30 min. each, as long as iodine is extracted.
7. 82A, 1 hr. Tissue may be stored in 82A for a considerable period if desired.
8. 95A, 1 hr.
9. 100A, 1 hr.
10. Ether-alcohol, 2 hr.
11. 2% celloidin, 3 days.
12. 6% celloidin, 1 wk.
13. Mount on block for sectioning.

14. Place block in chloroform, 4 hr., or until sufficiently hardened.
15. 82A, 2 hr. The block may be stored at this stage for as long as desired.
16. Section.
17. Place sections in 82A, 5 min.
18. 70A, 3 min.
19. Stain with Van Giesen's picrofuchsin, 5 min.
20. 70A, 3 min.
21. 82A, 3 min.
22. 95A, 5 min.
23. Cedar oil, 30 min.
24. Transfer section to a slide and mount in balsam.

Note the instructions for stains containing picric acid, page 349. Picrofuchsin will stain the celloidin; after observing the effect, carry a second section through step 22, then place the section on a slide and treat with 100A to remove the celloidin, thence through cedar oil to balsam.

*Paraffin Method:*

1. Fix a portion of small intestine in Bouin's fluid, 10 hr. or overnight.
2. Wash in several changes of 50A, 15 min.
3. Wash in several changes of 70A, 15 min.
4. 82A, 15 min. Store at this stage if desired.
5. 95A, 15 min.
6. 100A, 30 min.
7. 100A and xylene, equal parts, 15 min.
8. Xylene, 1 hr.
9. Xylene and melted paraffin, equal parts, 1 hr.
10. Paraffin bath 1, 30 min.
11. Paraffin bath 2, 30 min.
12. Paraffin bath 3, 30 min.
13. Imbed and section. The paraffin block may be stored indefinitely.
14. Prepare albumenized slides.

15. Transfer sections to flooded slides on warm plate and affix. Sections affixed to slides may be stored indefinitely.
16. After 24 hr. of drying, place slide in xylene, 15 min.
17. 100A, 1 min.
18. 95A, 1 min.
19. 82A, 1 min.
20. 70A, 1 min.
21. 50A, 1 min.
22. 35A, 1 min.
23. Delafield's hematoxylin, 30 min., or until section is decidedly blue.
24. Wash in water, 5 min.
25. 35% acid alcohol, a few seconds to a few minutes, until section is reddish in color.
26. 35% alkaline alcohol, until blue color is restored.
27. 50A, 1 min.
28. 70A, 1 min.
29. 82A, 1 min.
30. Eosin, 30 sec. to 1 min., until sufficiently stained.
31. 95A, 2 min.
32. 100A, 2 min.
33. Xylene, 5 min.
34. Mount in balsam.

## CHAPTER 15

### THE NEWER TECHNIQUES

*In This Chapter:* alternative and short-cut methods; improvements in the paraffin sectioning technique; dioxan, cellosolve, and others.

THE preceding chapters have taken up the standardized routines for sectioning, well known to all experienced microscopists for many years. Everyone who makes more than merely a passing study of microscopical technique sooner or later learns at the least the paraffin sectioning method, and has some knowledge of the principles involved in preparing material for the celloidin and freezing microtomes.

It is generally admitted that all of these procedures are at best lengthy, exacting, and tedious; yet they have been necessary in the past as a means to an end and as the only reliable techniques by which really adequate and worthwhile thin sections of organic structures could be obtained. Although most workers have been concerned with results more than with the means, there has always been a small body of research scientists to whom microtechnique for its own sake has appealed as a valuable subject for investigation; to them the constant improvement in method has been due.

Within recent years the annoyance and expense involved in procuring pure ethyl alcohol and the development of many new industrial reagents with remarkable powers of miscibility have led technicians to try out a large number of substitute chemicals and short-cut methods, the best and more generally accepted of which are here included. Some of these have to do mainly with dehydration; others with imbedding, or mounting; a few with all of these processes.



**Normal Butyl Alcohol.**—There are four forms of butyl alcohol, designated as normal (abbreviation, N or *n*), iso-butyl, secondary, and tertiary. The first and last are important commercially as solvents, *n*-butyl alcohol being used in large quantities in dissolving nitrocellulose to make lacquers. It was first proposed as a microtechnical agent by Mlle. Larbaud in 1921, and has since met with notable success in the hands of Stiles, Zirkle, H. M. Smith, and others. The process is not at all a short cut; rather it is slow and tedious, but has certain advantages that more than repay the extra trouble.

This alcohol is especially recommended for arthropods, woody tissues of plants, embryos, and organs that become too hard and brittle with the routine of ethyl alcohol-xylene treatment, such as spleen or liver.

For sectioning insects or ticks, Stiles used Gilson's as a fixer, slitting the chitin in one or two places with fine scissors to admit the reagents freely. His system, as slightly modified by Becker and Roudabush, proceeds from fixer to 70% iodinated alcohol to extract mercury, then into fresh 70A, 50A, and 35A, 30 min. in each. Next follows this schedule:

Container	Ethyl alcohol		Butyl alcohol, quantity, cc.	Time, hr.
	%	cc.		
1	50	90	10	2
2	70	80	20	2
3	83	65	35	4
4	95	45	55	6 to 48
5	100	25	75	3 to 12
6	100	25	75	3 to 12

Step 6 repeats 5, with fresh fluids, thence to straight butyl alcohol, two changes in the course of 3 or 4 hr. or longer. Objects may be stored in pure *n*-butyl indefinitely without overhardening, which may be an advantage at times in

fitting working schedules. Now the object is infiltrated with 2 parts paraffin to 1 of butyl in a covered dish in an oven, and then in paraffin alone for two or three baths over a period of 4 to 5 days. This slow infiltration is essential. Following this step, the material is imbedded in fresh paraffin and sectioned.

The advantages are a very slow but perfect infiltration without the usual overhardening, the object cutting more readily and perfectly. Several experts now use this method exclusively in preparing chick or pig embryos for sectioning.

**Tertiary Butyl Alcohol.**—Johansen reported in 1935 that this alcohol may be used for microscopical technique in the same way as detailed later for dioxan.

**Iso-propyl Alcohol.**—Propyl alcohol occurs in two forms, the normal and the iso-propyl, the latter often marketed under the term "isopropanol." Windsor tried out this reagent as a substitute for ethyl alcohol and found it excellent for both gross preservation and routine microscopy. More lately he reported isopropanol as distinctly better than ethyl alcohol with both the Gram stain and the Ziehl-Neelsen acid-fast techniques for bacteria. The advantage lies in the slower action. Destaining takes place at a slower rate so that a more exact and clear-cut differentiation is possible. The pure (100%, anhydrous) form is better for microtechnique than the commercial (about 95%) grade.

**Normal Propyl Alcohol.**—Clothier found that both iso- and *n*-propyl alcohols are splendid substitutes for ethyl alcohol and that they mix in all proportions with water, xylene, and cedar oil. They do not harden tissues to the extent met in using 100% ethyl alcohol; hence sectioning is easier and better.

**Acetone.**—This substance, a commercial solvent, has been used by occasional technicians both as a fixer and as a

substitute for ethyl alcohol in dehydration. Though not new to microscopy, it seemed best to include acetone at this point as a variant from the usual routine. Held made a 1% solution of mercuric chloride in a 40% acetone for fixing nervous tissue, and then washed out with increasing strengths of acetone. Bing and Ellerman fixed medullated nerves in a mixture of 9 parts acetone to 1 of formalin. Scholz fixed small objects of various sorts in warm acetone for 30 to 60 min. and then brought them directly into celloidin. After formalin fixation, acetone is particularly good as a substitute for ethyl alcohol: place the tissue in equal volumes of 10% formalin and acetone for 30 min., pure acetone for 2 hr., half acetone and half xylene for 30 min., pure xylene for 30 min. or until clear. Infiltrate with paraffin as usual. Acetone hardens tissues very rapidly, an objection that must be taken into account.

**Terpineol.**—Here is another reagent that avoids the use of strong ethyl alcohol. Terpineol will clear from an alcohol as low as 80%, and a mixture of 1 part xylene and 4 of terpineol has met with considerable recent use. Von Volkman employed this clearer in dehydrating sections affixed to slides, draining off water after the wash following aqueous stains and placing the slides directly in terpineol. After changing the terpineol once, slides were then put in xylene and thence to mounting in balsam.

**Anilin Oil-Methyl Salicylate.**—The general use of anilin oil alone to replace the higher alcohols and prevent overhardening has already been commented upon. Methyl salicylate is a synthetic oil of wintergreen. In combination, Becker and Roudabush recommend these reagents highly, after many trials. They fix in any desired mixture, as Zenker's, Bouin's, or Gilson's, and follow with the proper aftertreatment as in the ordinary technique, ending in 70A for 30 min. Then into 2 parts 70A, 1 part anilin oil for

2 hr.; 1 of 95A and 2 parts anilin for 2 hr.; pure anilin for 2 hr.; 1 part anilin and 1 part methyl salicylate for 2 hr.; methyl salicylate, two baths, 1 hr. each; paraffin, three baths, 1 hr. each; imbed and section.

**Dioxan.**—Probably the most important of all new micro-technical chemicals is dioxan, sometimes spelled dioxane. Chemically, it is diethylene oxide, a colorless, almost odorless, inflammable, slightly anesthetic, and poisonous liquid. It is a most remarkable industrial solvent and was first tried out in microscopy in Germany by Graupner and Weissberger in 1931; in this country by Mossman in 1933. Since then a great many technicians in all lines have worked with dioxan and are generally very enthusiastic, regarding this reagent as a revolutionary discovery, greatly simplifying and shortening the generally lengthy and tedious routines. It is miscible in all proportions with all of the reagents employed in paraffin sectioning—water, alcohol, xylene, paraffin, and balsam. Hence it may be used in any or all of the various steps performed in preparing tissues for sectioning or affixed sections for mounting.

The latest edition of Lee's *Vade-Mecum* regards dioxan as dangerous, stating that its steady use is apt to impair the health of laboratory workers. Toxic amounts (1:1,000 of air) may be inhaled without the individual's becoming aware of the risk, especially in small and poorly ventilated rooms, and the bad effects are cumulative. Usage should be restricted to advanced technicians and for special purposes.

On the contrary, Mossman, Guyer, and other American authorities minimize the poisonous properties of dioxan and believe that the small amounts used in microscopy will not prove at all harmful, if ordinary precautions are taken. Keep containers covered at all times when not actually transferring objects; do not needlessly breathe in the vapors or soak the hands in the fluid; where possible, have some

sort of ventilation in the room when working with this chemical. Navasquez found that the effects on experimental animals, far from being cumulative, showed the development of a rapidly increasing tolerance.

Reports from botanists have been somewhat conflicting to date as to the value of the dioxan technique in their field; some have used it with marked success, whereas others regard it as inferior. Animal histologists and cytologists are more uniform in their praise. A rough-and-ready dioxan-paraffin technique for ordinary animal tissues is to fix in 10% formalin, Bouin's or formol-acetic-alcohol, as usual, then transfer directly into 100% dioxan, of which there should be three vessels, marked dioxan 1, dioxan 2 and dioxan 3. Leave for 1 hr. in each of the first two, 2 to 3 hr. in the third; then in melted paraffin, also with three baths; 20 min. in the first, 40 min. in the second and 1 to 2 hr. in the third. Imbed and section. Mossman reports running through an organ from fixed tissue to finished slides in one day—certainly a vast saving in time.

As is nearly always the case, more gradual steps will give more perfect results. Wash the tissue in the usual manner following fixation, then pass through the three dioxan baths, as above, but go then to half-dioxan, half-melted paraffin, 2 hr., at 55 to 60° C. before proceeding with the paraffin baths. Sling the object on a strip of cheesecloth across each of the three paraffin vessels so as to support it somewhat above the bottom; the dioxan, being heavier than paraffin, will sink through and leave the paraffin unadulterated.

Fixing solutions may be made up with dioxan, but there seems to be no advantage gained by so doing and the results have not been always satisfactory. It is better to make up the fixers in the usual manner and begin the use of dioxan afterward, following washing.

After sections have been cut and affixed to slides, remove the paraffin by immersion in xylene; then go through two changes of dioxan into water. Stain as desired; rinse in

water; destain if necessary in a mixture of half water, half dioxan, plus 0.1% hydrochloric acid; alkalize with  $\frac{3}{4}$  dioxan to  $\frac{1}{4}$  water plus a bit of bicarbonate of soda; counterstain; rinse in 90 parts dioxan, 10 parts water; pure dioxan; two changes of xylene; mount in balsam.

The relation of dioxan to water as affecting dehydration on the one hand and the action of staining on the other is interesting and important, and must be taken into account in working with this solvent. The dioxan used for the baths preceding paraffin infiltration must be 100%, that is, free from water. Most technicians have found that the commercial grade is entirely satisfactory and requires no preliminary dehydration before use. Also, dioxan may be reclaimed for repeated use by removing any water carried over into it by tissues; for this purpose calcium oxide has proved better than calcium chloride. Some keep a few lumps of the oxide lying loosely in the bottom of the vessel; others make a platform of copper or brass screening, with the edges crimped down so as to support the tissue a bit above the bottom of the container, with the oxide lumps below. To test dioxan for purity, mix a bit with an equal amount of melted paraffin in a test tube and shake; if the resulting solution is clear, the dioxan is free from water.

Many of the commonly employed fixers are soluble in dioxan, hence one may pass from them into this reagent without washing, as is true with picric acid (*e.g.*, Bouin's fluid) and bichloride of mercury (*e.g.*, Zenker's or Gilson's). In most instances, the dioxan will extract mercury crystals so that no treatment with iodine is necessary. If with any particular object it appears from trial that such treatment should be given, dissolve some crystals of metallic iodine in the dioxan rather than use tincture or solution of iodine.

In the case of stains, however, most of them are insoluble or but slightly so in pure dioxan, and the addition of 10% water to the solvent enables the majority to dissolve readily. Counterstains should hence be applied in solutions

of 90% dioxan, 10% water. Differentiation is very easy on this account, as one has only to place the slide in pure dioxan to halt the extraction of a dye. For example, if eosin has been selected as the counterstain, it is applied as a 0.5% solution in 90% dioxan, 10% water, until the section is well stained. Then the slide is placed in 90% dioxan, 10% water to rinse and differentiate. As soon as the correct intensity of the eosin is secured by this destaining action, transfer the slide to pure dioxan, whereupon extracting of the stain is stopped.

Avoiding the use of xylene entirely, one may dissolve solid balsam in dioxan and mount in this mixture. We have not tried this procedure long enough, at the present writing, to be able to pass judgment on its comparative merits, but it seems to be perfectly satisfactory.

Dioxan works beautifully with either the freezing or paraffin method of sectioning, but so far has not been adaptable to the celloidin technique. Objects may be stored in dioxan indefinitely without any apparent harm. Mossman stored some tissues in dioxan for nearly 3 yr. and found that they sectioned and stained perfectly at the end of that period.

**Methyl Benzoate.**—Some technicians like to use this substance for its remarkable clearing powers, though it is somewhat expensive. Objects clear so beautifully that the benzoate serves as an indicator to show how complete the dehydration has been. Pass from absolute alcohol into two baths of methyl benzoate, 12 to 24 hr. each; thence to pure benzene, two changes, 30 min. each; benzene and paraffin, equal parts, 1 hr.; to pure paraffin. If the object would be injured by 100A, it is permissible to transfer from 95A into the benzoate, which will not harden tissues. This substance is also used as a substitute for cedar oil with immersion objectives; its refractive index is very similar to that of cedar oil and it does not have to be wiped off of the objective, since it will evaporate.

**Trichlorethylene.**—Oltman, in 1935, recommended this chemical as a substitute for xylene throughout the usual paraffin technique, including both sectioning and staining operations. Mount in balsam which has been dissolved in this reagent instead of in xylene. Slides dry very quickly.

**Cellosolve.**—Another recent industrial solvent which is making rapid strides in adoption by technicians is this ethylene glycol mono-ethyl ether, given the trade name cellosolve. First reported by Frost, 1935, in England, it is being taken up by many American workers who are enthusiastic over its time-saving advantages. As detailed by Hance, the steps in cellosolve technique are to fix and wash tissue as usual, then dry the object with a piece of paper toweling and place in cellosolve, where it may remain indefinitely, thus accommodating easily to all sorts of time schedules. The minimum stay in this solvent is 2 to 4 hr., with one change to fresh cellosolve during that time. Pass the tissue through two changes of xylene during 15 to 30 min., thence to paraffin infiltration, imbedding, and sectioning. When the sections have been affixed to slides, remove the paraffin with the xylene as customary, then place the slide in cellosolve, two jars, for 5 min. each; distilled water, stain, rinse, destain, and rinse as usual; then two jars of cellosolve for 2 min each; counterstain, with the dye dissolved in cellosolve; clear in a mixture of oil of cloves and oil of thyme; xylene; mount in balsam.

The whole alcohol series is thus avoided throughout the entire technique, effecting a saving in time and materials and greatly simplifying the procedures.

**Tergitol Penetrant No. 7.**—Hance also reports on the use of this wetting agent which, by lowering the surface tension of chemicals with which it is mixed, speeds up the penetrating power of all reagents tried thus far by several times. This is of considerable importance in fixing solutions where rapid penetration is essential to prevent post-mortem



changes. Tergitol in albumen fixative spreads paraffin ribbons more rapidly and perfectly, using less heat, and also speeds up the action of staining.

Not more than 1% should be added to fixing solutions, and only one drop to the ounce of Mayer's albumen fixative or to any staining jar, such as a coplin jar of hematoxylin.

**New Imbedding Mixtures.**—Hance's rubber-paraffin-beeswax mixture, published by him in 1933, has now met with wide acceptance. It is somewhat difficult to prepare but gives a medium of much better and more reliable consistency which overcomes the tendency of pure paraffin to crystallize. Chop up 20 g. of crude sheet rubber, either smoked or unsmoked, and dissolve in 100 g. of smoking-hot paraffin, with constant stirring. When cool, put in a container marked as Hance's rubber-paraffin stock. To prepare the imbedding medium, add 4.5 g. of this stock and 1 g. of beeswax to 100 g. of commercial parawax. Melt these ingredients together and filter through paper toweling.

More lately (1939), the same author discusses the advantages of adding substances to various paraffins or paraffin mixtures that will lower the melting point. Such additions increase the thoroughness of penetration with less injury to the object and also are aimed at overcoming the liability of pure paraffin to crystallize. From 2 to 5% of lanolin or hard sheep fat added to paraffin or to the rubber-paraffin-beeswax mixture will accomplish these results and protect the tissues from excessive hardening during infiltration. Lanolin is a fatty substance prepared from sheep's wool and is miscible with water to a certain extent, so Hance has tried a nondehydrating technique of passing tissues, after fixing and washing, directly into melted lanolin, 5 to 6 hr., followed by three or four changes of lanolin with 4 hr. in each dish. Remove the object to paraffin to which 2 to 5% hard sheep fat has been added and infiltrate for 2 to 3 days. This novel technique is slow and uncertain, sometimes giving remarkable success, at other times failure.

Readers may wish to experiment with it and see what they can do with a method that avoids the whole series of alcohols and xylene.

Another mixture, which has been recommended for paraffin imbedding, is that of Altmann, who takes 85 g. of paraffin of 53 to 55° C., 10 g. of stearin and 5 g. of beeswax, thoroughly melts and mixes them, then filters. Store as a cold solid and remelt for use.

**Synthetic Neutral Mountant, F & G.**—As a substitute for Canada balsam or gum damar in mounting slides, Flatters & Garnett, Ltd. have produced a synthetic resin that is neutral and will not crystallize. This mountant is almost colorless and, as far as we have been able to determine in the short time during which trial slides have stood, remains so indefinitely. Damar yellows slowly and balsam rapidly, and both are complex resinous mixtures with definitely acidic properties. Even though neutralized at the time of mounting slides, both of these natural resins will shortly revert to an acid reaction and affect stains adversely. The synthetic neutral mountant will not do so. It is soluble in xylene and so may be thinned with this oil if the stock mountant becomes too thick after standing in the applicator bottle.

**Isobutyl Methacrylate.**—This recent du Pont product makes a water-white mountant that hardens in 5 to 10 min. and will not turn yellow with age. O'Brien and Hance suggest that it take the place of both mountant and cover glass. They dip a stained section into a thin solution of this reagent and withdraw it carefully so that the fluid drains evenly from the slide. They find that the thin film remaining hardens rapidly and forms as good a protection for the material as would the ordinary cover glass. It will stand up under thorough polishing with a paper handkerchief and can be written on with ordinary ink, supplanting a paper label. Or, if preferred, the paper label may be written in the

customary manner, dried and affixed, then coated with the methacrylate along with the sectioned object, thus giving the label a protective coating. The back of the slide may be left covered with the solution or may be cleaned with a cloth moistened with benzene or xylene, which are the solvents used in preparing this resin. These authors have also employed isobutyl methacrylate in filling deep-well mounts, since it dries so quickly. The low refractive index (1.477) may be a disadvantage in some cases.

**The Clarites.**—Two new mounting media, clarite (formerly known as Nevillite V) and clarite X (formerly-Nevillite No. 1) have been investigated by Groat, who describes them as cycloparaffin polymers, water white in color, inert, high-melting, and absolutely neutral. Clarite is slightly more expensive, has an R. I. of 1.544, and does not discolor at all with age; clarite X is somewhat cheaper, has a higher R. I. (1.567), and yellows slightly on standing, though much less so than either damar or Canada balsam. Both may be secured as lump resins or as solutions in xylene, toluene, or benzene. A 60% solution in any of these solvents is recommended, being thinner than the usual balsam, yet solidifying more rapidly. Groat preferred toluene as the best solvent whereas the technicians at the General Biological Supply House found xylene much more to their liking. They state that toluene-clarite dries too rapidly, forming crusts when first applied and sucking air bubbles under the cover; the slower drying xylene does not show these defects so markedly.

Unless the slightly higher refractive index of clarite X is desirable, the regular clarite should be chosen for its absolute transparency, which seems to be lasting. Another important advantage over balsam is the neutrality of clarite, with the consequent greater permanence of stains. We shall not be surprised if this synthetic medium, or something similar, replaces natural mountants entirely in the coming years. The clarites are soluble in dioxan and

may be so prepared when using the dioxan technique, if preferred.

**Plastic Covers.**—Necessity has been, at least partially, the parent of a very recent invention designed to supplement or replace the cover glass. Conditions in Europe have made the importation of these glasses into America difficult or impossible; also they are always expensive and fragile. Professor Hance has been working with a form of plastic material adapted for this use and calls them "Histo-covers:" type A is for cemented mounts; type B for water mounts. At the present writing he is supplying these covers, including the cement with type A (see Appendix A); we have also received samples of similar covers from the General Biological Supply House.

Hance believes these plastic slips superior to glass and feels that they will eventually supplant the older article. He recommends that all microscopists give them a trial.

#### SPECIMEN SCHEDULES WITH SOME OF THE NEWER TECHNIQUES

##### *Dioxan*

Kill a rat with illuminating gas or by drowning and remove the spleen entire. Slit spleen lengthwise with a safety-razor blade and rinse each piece for a moment in normal saline.

Fix in Gilson's mixture to which 1% tergitol has been added, 2 hr.

Wash, running water, 6 hr.

Blot each half for a moment with paper toweling and place in 100% dioxan, 1 hr.

100% dioxan (fresh jar), 1 hr.

100% dioxan (fresh jar), 2 hr.

Dioxan-paraffin, half-and-half, 2 hr. Add 4% lanolin to the paraffin used.

Paraffin plus 4% lanolin, infiltration 1, 20 min.

##### *Cellosolve*

Kill a small salamander with ether and remove both ovaries. If the specimen is a male, try others until a female is secured. Slit each ovary lengthwise with a safety-razor blade and rinse each piece for a moment in normal saline.

Fix in Bouin's fluid, 6 hr.

Blot pieces for a moment with paper toweling and place in cellosolve, 2 hr.

Cellosolve (fresh jar), 2 hr

Xylene 1, 10 min.

Xylene 2, 10 min.

Xylene plus rubber-paraffin-beeswax mixture, half-and-half, 30 min.

*Dioxan*

Paraffin-lanolin 2, 40 min.  
 Paraffin-lanolin 3, 1 hr.  
 Imbed in paraffin-lanolin and section on a paraffin microtome.  
 Affix sections to slides, adding 1 drop tergitol to 1 oz. of Mayer's albumen fixative. Spread on a warm plate then store slides for 24 hr. before staining.  
 Xylene 1, 10 min.  
 Xylene 2, 10 min.  
 100% dioxan 1, 10 min.  
 100% dioxan 2, 10 min.  
 Distilled water, 5 min.  
 Stain in Delafield's hematoxylin plus 1 drop tergitol, 10 min.  
 Wash, tap water, 5 min.  
 Destain in 50% dioxan, 50% water, plus 0.1% hydrochloric acid, 30 sec. to 5 min., until section appears reddish.  
 Transfer to 75% dioxan, 25% water, plus few drops of 0.1% aqueous solution of bicarbonate of soda, until section again is blue.  
 Counterstain in picrofuchsin, 5 min. Make up this stain using dioxan as the solvent instead of water.  
 Differentiate in 90% dioxan, 10% water, 1 to 5 min., until stain is satisfactory.  
 100% dioxan, 10 min.  
 Mount in a solution of 60% clarite, 40% pure dioxan.

*Cellosolve*

Rubber-paraffin-beeswax, infiltration 1, 1 hr.  
 Rubber-paraffin-beeswax 2, 1 hr.  
 Rubber-paraffin-beeswax 3, 30 min.  
 Imbed in rubber-paraffin-beeswax and section on a paraffin microtome.  
 Affix sections to slides. Spread on a warm plate and store slides for 24 hr. before staining.  
 Xylene 1, 10 min.  
 Xylene 2, 10 min.  
 Cellosolve 1, 5 min.  
 Cellosolve 2, 5 min.  
 Distilled water, 5 min.  
 Stain in Ehrlich's hematoxylin, 30 min.  
 Wash, tap water, 5 min.  
 Destain, if necessary, in water plus 0.1% hydrochloric acid (aqueous destain).  
 Alkalize with a water rinse containing a few drops of 0.1% bicarbonate of soda.  
 Cellosolve 1, 2 min.  
 Cellosolve 2, 2 min.  
 Counterstain in cellosolve saturated with orange G, 5 min., or until well stained.  
 Clear in mixture of equal parts oil of thyme and oil of cloves, 10 min.  
 Xylene, 10 min.  
 Mount in Synthetic Neutral Mountant, F & G.

## CHAPTER 16

### SPECIAL PREPARATIONS

*In This Chapter:* permanent slides demonstrating hard woods, cell walls, elastic tissue, fat, decalcified bone, mitosis, giant chromosomes, condriosomes, Golgi apparatus, nerve cells and fibers, embryos of frog, chick, and pig; osmic acid fixation, serial sectioning, anatomical and micro-injections.

**I**N THE immediately preceding chapters most of the methods were general, that is, applicable to a wide variety of circumstances. One may fix liver, intestine, kidney, and so on in a great many fluids, such as formalin, Gilson's, Bouin's, Zenker's, and others, with little to choose from between them. Sections may be prepared by any method, and almost any combination of stains will serve, as long as one is basic, for nuclei, the other acid, for cytoplasm, and the two with contrasting colors. No one would think, for example, of employing safranin and Congo red together, since these are both red dyes. The choice of fixer, stain, and clearing agent is often a matter of individual preference.

There remain, however, a number of structures which require special treatment and which cannot be processed by routine methods; otherwise, they will not reveal the specific parts for which the work is performed. The present chapter will take up a number of such cases, referring you to more comprehensive manuals for more elaborate treatment or further examples.

**Hard Woods, Nuts, Shells.**—It is customary, in order to secure sections of stem and root of various plants for structural studies, to select young and green shoots and cut them by regulation microtome methods, taking advantage

of the stage before they become too hard to cut. However, old and hard woods present a picture that cannot be neglected; they require a special technique.

First saw the wood into small blocks, of a size suitable to

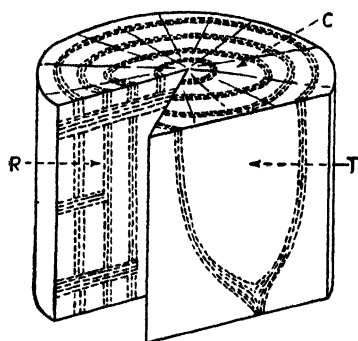


FIG. 119.—The three planes of sectioning wood. C, cross or transverse; R, radial; T, tangential.

mount on slides, taking care to follow one of the three planes used in such studies (Fig. 119). These are the cross or transverse section, at a right angle to the longitudinal axis of the tree stem; the radial section, which is longitudinal and lies in the plane of a radius passing from center to margin, like the spoke of a wheel; and the tangential section, longitudinal but in

the plane of a tangent, to one side of the center. Carefully avoid oblique sections that are not in one of these three planes, as they are very confusing.

Boil such a block for 24 hr. in tap water. For pine and woods of similar softness, this is sufficient. Woods of intermediate hardness are next treated by soaking for 1 wk. or longer in equal parts of 95A and glycerin; really hard woods, such as oak, after the boiling in water, are placed in a 25% hydrofluoric acid for a week, washed thoroughly in tap water, then put in the alcohol-glycerin mixture for 1 wk. or more.

*Caution:* Hydrofluoric acid is a very powerful reagent and will corrode glass. It must be kept in a paraffined container and, needless to say, must be handled with caution. Since even the fumes of this acid are dangerous and will damage microscope lenses, do not work with hydrofluoric in the same room in which you keep your microscope.

The soft woods may now be cut on a regular microtome; the celloidin method is best, but the others are satisfactory. The harder woods should be sliced while a jet of steam is

playing over the specimen as it is held on the microtome block. This is not difficult to arrange. A flask of water is boiled on a ring stand over a bunsen burner. The neck of the flask is provided with a two-hole rubber stopper, one for a thistle tube that reaches nearly to the bottom of the flask, the other for an outlet tube, beginning just below the level of the rubber stopper and bent to lead to a point immediately above the wooden block being cut. New water is added to the flask as needed, through the thistle tube. It is recommended that the temperature of the steam be about 90° C. If much lower, no advantage is gained; if much higher, the wood is dried out to an unsatisfactory state.

Nut shells and fruit stones may be cut on the microtome by using this same steam technique.

**Cell walls** are most interesting when impregnated with silver; for this purpose, the reagent used is silver nitrate. One preparation recommended in several manuals is to remove the eye of a frog or other convenient animal and quickly rub over the cornea a piece of silver nitrate (in the solid state). Slice off the cornea, place in distilled water in a watch glass, and with a camel's-hair brush remove the epithelium. Expose the dish to sunlight or at least strong daylight until the preparation appears brown. Wash in fresh distilled water, dehydrate, clear, and mount in balsam. The silver nitrate penetrates the epithelial layer and soaks into the fibrous tissue, where it is reduced by the action of light in the same way as on an exposed photographic plate, and metallic silver is deposited in the cell walls. Since the cells themselves are unstained, many prefer to treat the preparation with hematoxylin in the usual way to bring out the nuclei in addition to the cell walls.

Various membranes, treated successfully in similar fashion, yield exceptionally beautiful results, but failures are to be expected with any impregnation method. If a frog is kept for some time in an aquarium of water, pieces



of shed skin (epithelium) will be found floating in the water; the lining membrane of the abdominal cavity (mesothelium) and of large blood vessels (endothelium) are all excellent for mounts showing cell walls. The preferred method for endothelium is to inject with a syringe one of the large arteries leading from the aorta across the supporting mesentery to the intestine. Open the main vein draining this region and inject 1% silver nitrate in distilled water into the artery until this solution runs from the vein; after 2 min., follow with distilled water again to wash out excess nitrate. Remove this section of the artery and surrounding mesentery, slit open the artery in a dish of distilled water, spread out the membrane, and expose all to sunlight, under water, until brown. Rinse in fresh distilled water, dehydrate, clear, and mount suitable portions in balsam.

Fresh membranes may be treated without previous injections if they are stretched tightly, like a drumhead, over or between objects. Guyer uses two small vulcanite rings, one fitting rather closely within the other, stretching a piece of the membrane just as one does a cloth between embroidery rings. Such a stretched membrane is first washed well with distilled water, then treated with 1 part of silver nitrate in 300 parts of distilled water, in direct sunlight, and constantly agitated while exposed, until the tissue browns or darkens. Then wash in fresh distilled water, remove from the stretching device, dehydrate, clear, and mount in balsam. The stretching is essential to prevent impregnation of every fold and wrinkle; the preliminary washing does away with foreign matter that might stain. Inspect the finished preparations carefully, as some parts should show details well, though several trials may be necessary to get a really fine slide.

**Elastic tissue** is stained with orcein or resorcinfuchsin. Stain sections of the aorta or other large artery from 95A in orcein for 30 min. to 1 hr., or in resorcinfuchsin for

15 to 30 min., wash in 95A, and differentiate (if necessary) in 95% acid alcohol, rewashing in 95A, dehydrating in absolute alcohol, clearing, and mounting in balsam. Orcein stains the abundant elastic fibers dark brown; resorcin-fuchsin stains them dark blue. If a counterstain is wanted for nonelastic connective tissue, picrofuchsin is recommended.

**Fat** requires a specific stain and cannot be processed through alcohols, since these dissolve out the oil itself and leave nothing but empty fat cells. Two chief methods obtain: treating with osmic acid as a fixer, which blackens fat (olein), or staining with either Scharlach R or Sudan III, both specific for fat and imparting an orange to red color. Osmic-fixed material may be sectioned by the paraffin method and dehydrated and mounted in balsam if 100A and xylene are not used and cedar oil substituted for final dehydration and clearing. Frozen sections may be stained with Scharlach or Sudan and mounted in glycerin jelly.

One of the most interesting preparations, however, is made as follows: Cut out a bit of mesentery from a freshly killed mammal, about  $\frac{3}{4}$  in. square, and include a minor blood vessel with a few of its branches, and stretch it with needles in the center of a slide; some prefer to make the preparation on a cover glass instead. Keep the center moist by breathing on it, at the same time allowing the four corners to dry so as to affix the stretched membrane to the glass. Invert the slide over a small bottle mouth so the tissue will be bathed in the fumes of formalin for 15 min. This is vapor fixation and works well in some cases, as here. Mix up 50 cc. each of acetone and 70A and dissolve in this all of the dry powder of Scharlach R that will go into solution (saturated solution). Filter this stain and apply it in a tightly closed vessel for 10 min. or until the fat cells are all well stained, rinse in 70A, wash in water, and counterstain with 1% aqueous solution of methylen blue (do not confuse with methyl blue, which is a different stain), wash in water, and mount in glycerin jelly.

**Decalcified Bone.**—The preparation of dry ground bone was taken up in Chapter II, a method for presenting a black-and-white picture of bone structure in which the mineral elements are retained while the organic parts are removed. The other technique on bone is just the reverse, to destroy the mineral parts by a process termed “decalcification,” but preserve the organic structure. Decalcified bone is rendered sufficiently soft so as to cut on a microtome, as in the preparation of sections of other tissues.

Saw a short section,  $\frac{1}{4}$  in. or less, out of the femur of a freshly killed cat or rabbit, to prepare cross sections, and from the other side of the body a sagittal section through the head of the femur (bisecting the head lengthwise) to show a longitudinal section as well as the interesting structure where head and neck of femur spring from the main shaft. Carry each piece separately through the following technique. Fix for 24 hr. in any of the better fixing mixtures, as Zenker's or Bouin's, and follow with the appropriate washing treatment. Place in about forty to fifty times by volume of nitric decalcifier for 1 wk., or until the bone may be pierced readily with a needle. Do not apply this test for softness at a place where sections are desired, as it mutilates the tissues. During decalcification, shake the vial several times a day to ensure thorough penetration. Wash well in 70A, two changes, 24 hr. each, to remove all acid, then 82A for 30 min., 95A for 30 min., 100A for 1 hr., cedarwood oil until clear, but no longer than necessary; rinse in xylene 2 to 3 min., infiltrate and imbed in paraffin, using minimum heating periods; then section. Many workers prefer the freezing method; probably the best slides of bone are made by the celloidin method. Use these if you have the equipment.

Almost any regulation staining combination may be employed, as hematoxylin and eosin, or alum cochineal and light green. Mallory's triple stain makes a very beautiful slide.

Sections of decalcified teeth are prepared in the same

manner, and it is better, in the case of longisections, to leave the tooth in place in the jaw and section both together.

**Mitosis.**—Favorite materials for cell division studies and the observation of chromosomes include root tips of onion, iris, hyacinth, and tradescantia; anthers and ovaries of the lily, among plants; spermary of crayfish, grasshoppers, and salamanders; and eggs of ascaris and whitefish among animals. The last two present such difficulties that they are not at all suited for the inexperienced, but advanced workers will know where to look for detailed technique references. We shall limit descriptions here to onion root tips, typical of the procedure for all other ordinary materials.

Place an onion in a vessel of water and allow it to remain until it has put forth numerous roots. The extreme tip of a new root is then severed to give a length of about  $\frac{1}{4}$  in., and several such pieces are placed in the fixer. For some years it has been known that cell division does not occur with equal rapidity at all hours and that, in the onion, mitoses take place most numerous a little before midnight and somewhat after noon, the hours of 11 P.M. and 1 P.M. being best for removal and immediate fixation. One of the famous Flemming mixtures is generally considered best for fixing, though Bouin's fluid is also excellent. Although most of our readers will use Bouin's, we include here a description of the Flemming fixation for the benefit of those who may wish to try it. The Flemming formulas include chromic, osmic, and acetic acids in various proportions and are hence often termed the "chrom-acetic-osmic fixers," the greatest drawbacks of which are the expense and danger attending the use of osmic acid, a very poisonous chemical costing in the neighborhood of \$6 per gram.

Osmic acid (osmium tetroxide,  $\text{OsO}_4$ ) is furnished in sealed glass ampules. One of these is first soaked in water to remove the label, then rinsed thoroughly, and placed in a small glass-stoppered bottle containing 100 cc. of distilled water. The acid is highly volatile and extremely poisonous

in all forms—the solid crystals, solutions, or vapor. It becomes reduced when in the presence of the slightest trace of organic particles, hence all glassware and instruments used when working with or storing this reagent must be scrupulously clean and always free from dust of any sort. Having seen to it, then, that the glass ampule, the bottle, and the distilled water all meet this requirement for cleanliness, break the ampule with a clean glass rod, apply the stopper immediately, and wash the glass rod under a tap of running water. Be sure to label such a bottle carefully to avoid hazards.

This makes a 1% aqueous solution of osmic acid, which provides a stock from which to make up mixtures. To prevent possible loss through reduction of this valuable chemical, Lee recommends keeping the osmic solution in a chromic acid solution and uses a 2% osmic in a 1% chromic. To make this, first prepare a 1% solution of chromic acid crystals in distilled water, then to 50 cc. of this add 1 g. of osmic acid by putting in a 1-g. ampule and breaking as before directed; with a glass rod. This, too, provides a stock solution from which to make up various combinations. It is important to note with the Flemming mixtures, the formulas for which are given in Chapter 17, that the acetic acid is never added until immediately before the fixer is to be used, since the complete fixers do not keep well. Chamberlain dissolves chromic and acetic acids together as a stock solution *A* and the 1% osmic separately as solution *B*, mixing the two just before using. He finds, after a long series of trials on onion root tips, that a proportion termed the Chicago formula works best on this plant.

Fix for 24 hr. and wash in running water for 12 hr., then proceed according to the regular paraffin technique, cutting sections 5 to 6 microns thick. The same applies also to Bouin fixed material. Stain in iron hematoxylin; bleach in peroxide, dehydrate, clear, and mount in balsam. Use a No. 1 cover glass if the sections are to be studied with an oil immersion lens.

An excellent alternative staining technique is the safranin-gentian-orange combination, which employs saturated aqueous solutions of safranin and gentian violet and a clove oil solution of orange G. With the sections cut and on slides, run them down to distilled water, then stain in safranin for 24 to 48 hr., rinse in water, and stain for an average time of 5 min. in gentian violet. A few trials will determine the most satisfactory time for this stain. Now rinse in 95A and put on oil of cloves containing a 1% solution of orange G for 1 to 2 min., after which the section is put in xylene, then mounted in balsam. The orange-clove oil reagent not only applies the orange stain but acts as a differentiator for the safranin and violet, extracting them; hence this step requires some practice for proper control. Some technicians prefer to differentiate with absolute alcohol, following the rinsing in 95A; then to add the orange in the form of a saturated aqueous solution, applying with a pipette for only a moment; then rinsing in 100A and passing quickly through oil of cloves to xylene.

The simplest staining combination for demonstrating chromosomes is the regulation safranin-light green technique, as recommended for many other botanical preparations. Stain in safranin, according to the preceding schedule, and dehydrate to 95A. Counterstain with a 0.5% solution of light green in 95A, rinse in fresh 95A, and proceed to complete the slide as usual.

A very beautiful slide may be made by staining the chromosomes with gentian violet, the cytoplasm with orange G.

**Chromosome Smears.**—A recently discovered preparation that has been causing a great deal of excitement in biological circles is a smear slide of the giant chromosomes found in the salivary glands of certain fly larvae, as *Drosophila* and *Sciara*. Although scarcely to be considered as elementary, this technique is within the capacity of readers who have progressed satisfactorily to this point.

*Drosophila* has become the most famous of all animals in genetics. It is a very small fruit fly, to be seen in cities or villages during summer months, hanging motionless or darting back and forth on extremely rapid wing beats in the vicinity of exposed fruits at the markets; the females attempt to deposit their eggs there, particularly on slightly decaying bananas. Expose a bottle of prepared food at a fruit stand, and there will be a number of females in it after a short time; you should then plug the bottle with cotton and remove it to your laboratory for further work.

One of Bridges' formulas for cultivating *Drosophila* is to dissolve 40 g. of agar in 2,000 cc. of water by bringing to a boil, at which time 200 g. of corn meal are added slowly with continual stirring. When well mixed, add 140 cc. of Karo corn sirup and 140 cc. of Bre'r Rabbit molasses and allow to cook slowly for 10 min. Do not overcook or the mass will become too hard to be of use. Remove from the fire and pour at once into culture bottles, for which purpose half-pint milk bottles have become standard. Fill the bottles to a depth of about 1 in.; when cool, punch a hole with a pencil butt, at one side, clear to the bottom, inserting a short section of glass tubing in this hole so that gases caused by fermentation can escape.

Take a fresh yeast cake and stir up in a little water to make a suspension. Add one drop of this to the top of the cold food mass in the bottle. Put a piece of paper toweling on top of the food and then add the flies. A bottle of this sort will support up to 18 to 20 pairs of these small insects for 2 to 3 weeks, at the end of which time they should be changed to a fresh bottle. To remove adult flies, etherize them until stunned, then dump them out upon a sheet of paper to sort or examine for characteristics. Handle with a fine forceps and replace in bottles before they recover. Bottles are plugged with cotton, which admits sufficient air.

The females captured at the fruit store, or purchased from a biological supply house, will soon lay eggs which will hatch in a few days, providing you with the larvae

wanted in this work. Fully mature larvae will crawl up the glass sides or upon strips of paper toweling in order to pupate; only these larvae should be selected for chromosome smear mounts.

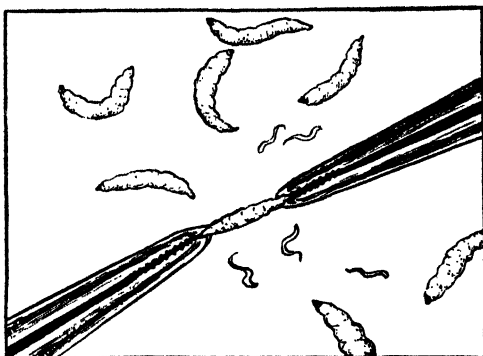


FIG. 120.—Method of using two forceps to pull a *Drosophila* larva in two. Most specimens break just behind the head, revealing the salivary glands—the smaller spiral structures. Operation done in water under low magnification. (Drawn from a photomicrograph by R. W. Cumley in *Educational Focus*, Bausch & Lomb.)

A wide-field binocular microscope is highly desirable, but other types may be substituted. Use a dissecting microscope with a  $10\times$  magnifier, or remove the front lens combination of the low power objective of your compound instrument. The remaining lens gives a low magnification at a high elevation, providing working distance. Place several mature larvae in a watch glass with Ringer's solution for cold-blooded animals, or in normal saline. Grasp one with two pairs of fine forceps, one at each end, and pull the animal apart; or tease the head end off with a needle while holding the maggot down with another needle or forceps. Usually the larvae will break just behind the head region and the salivary glands come away, as was done in making whole mounts from the grasshopper or roach. The glands are elongate, curved, minute, whitish bodies, two in number (Fig. 120).

Place the glands in 45% aceto-carmine, 15 min. Transfer them to a slide which has had a very light coating of



albumen fixative that has been allowed to become dry. With filter paper, remove any excess stain that may be present and add 50% aceto-carmin. Rub a finger along the side of the nose or through the hair to obtain a fine film



FIG. 121.—Giant salivary-gland chromosomes from larva of *Drosophila melanogaster*, 600 X. Compare their size with the gonadial chromosomes shown inset, upper right, which were drawn from a slide at 900 X magnification. (*Educational Focus*, Bausch & Lomb.)

of oil, and apply this to one surface of a cover glass. Mount this glass, oily side down, on the salivary glands and press on them gently so as to mash them out and thus isolate and display the various cells with their nuclei. Blot away any excess stain and put the slide for 12 to 24 hr. in a desiccator with a vessel of 95A, or strips of paper toweling saturated with 95A, so as to expose the slide to the fumes of this alcohol. Remove the slide to a dish of 95A to soak off the cover; if it does not come away after a while, pry it off gently with a toothpick. Mount the preparation in euparal or diaphane. Nuclei should be dark colored; cytoplasm of cells pinkish; chromosomes carmine. Several trials may be necessary before success is attained.

In most parts of the body where the chromosomes in the nuclei have been studied, they are condensed and extremely minute, appearing as a solid mass under any known stain and showing no internal details whatever. In the salivary glands, however, the chromosomes are more than one hundred times as large and are readily studied without oil immersion, revealing many alternating bands of dark and light color (Fig. 121). Interest centers around these bands, which some think may represent the actual genes that transmit hereditary characters; at all events, certain bands are known to be definitely associated in some manner with certain genes.

**Chondriosomes (Mitochondria).**—These minute granules in many plant and animal cells were largely overlooked by early workers since the usual fixing procedure destroyed them and special methods are necessary for their demonstration. A great many techniques have been recommended and the literature on the subject is fairly extensive. The following is one of the simplest methods. Onion root tips again are widely used to show mitochondria; for animal tissues, the pancreas is perhaps the best organ. Neutralize some 10% formalin, enough to serve as a fixer for the material chosen, by adding magnesium carbonate until more than saturated, a deposit of the carbonate remaining in the bottom of the dish. Fix in this for 48 hr., wash in 50A for 6 hr., proceed by the paraffin method, and stain with iron hematoxylin. Nuclei and chondriosomes should stain black; the Golgi apparatus (see next topic) usually remains unstained.

A longer but much better and more certain procedure is the Regaud technique. Prepare a quantity of 3% solution of potassium bichromate and have at hand some full strength commercial formalin. When ready to fix, using only small pieces of tissue, take 4 parts of the bichromate to 1 of formalin (*e.g.*, 80 cc. of bichromate, 20 cc. of formalin) and mix together. Fix in this mixture in the dark for 4

days, changing to fresh fixer every day. Do not add the two ingredients until ready to use, as formalin reduces the bichromate and the mixture will not keep.

Mordant for 1 week in the 3% bichromate, changing to fresh reagent on the second, fourth, and sixth days; wash, 24 hr., in running water, then proceed with dehydration, clearing and imbedding by the paraffin schedule. Cut sections 5 microns thick, mount on slides, hydrate, and form water stain as follows: mordant in 5% iron alum, 24 hr.; rinse briefly in distilled (not tap) water; then stain, 24 hr., in a hematoxylin made by dissolving 1 g. of hematoxylin crystals in 10 cc. of 100A, then adding 10 cc. of glycerin and 80 cc. of distilled water. This stain must ripen for about one month before use. Differentiate in the mordant, 5% iron alum, controlling by repeated observations under the microscope until the intensity of the stain is correct.

**Golgi Apparatus.**—This structure of certain animal cells and the possible equivalent in plant cells is, like mitochondria, of comparatively recent discovery and requires special treatment. Occasionally some form of simple technique using a formalin fixer is recommended, but generally such slides are worthless or constitute lucky accidents. A rather difficult technique using an osmic acid fixation or silver nitrate impregnation seems essential in order to secure really good or dependable results. Ludford's method, as follows, is one of those preferred today: Fix small pieces of pancreas or spinal ganglion for 18 hr. in a mixture of equal parts of 1% osmic acid and a saturated solution of mercuric chloride in physiological salt solution. Wash in distilled water for 30 min. Osmicate in 2% for 3 days at 30° C.; by which is meant placing in 2% osmic acid, just enough to cover the tissues, in an incubator. Reduce in water, 1 day, at 30° C. Then dehydrate, clear, imbed in paraffin, cut sections 5 microns thick, affix to slides, remove paraffin, and mount in balsam without any staining, the osmic fixation accomplishing the blackening

of the Golgi apparatus. If a counterstain is preferred, run the slides down to water and stain in a solution consisting of 1,000 cc. of distilled water, 2 cc. of 1% glacial acetic acid, and 1 g. of neutral red for 30 sec. or less. Rinse off excess stain with distilled water, shake off remaining water, pipette on 100A to differentiate and when correct, clear in xylene, and mount in balsam.

Not infrequently it may happen that osmication blackens not only the Golgi apparatus but the mitochondria and fat globules or yolk as well. An excellent differentiator has been found in turpentine, which will progressively remove the black, first in fat and yolk, then in mitochondria, and finally, after prolonged treatment, in the Golgi apparatus. Hence, when such conditions are found, it is only necessary to remove a second slide from xylene, just before mounting in balsam, and place it in turpentine for about 15 min. Inspect under the microscope from time to time, and check the action, when other bodies have faded but the Golgi apparatus is still black, by passing back through xylene and mounting in balsam.

**Nerve Cells.**—Nervous tissue as a whole requires special methods, often of great complexity. So advanced has this technique become that an entire separate science, neurology, is devoted to the study and its attending technique. A few of the major examples are given here; for further and detailed treatment, consult the larger and more advanced manuals.

*Nissl bodies*, or tigroid substance in nerve cells, are demonstrated by Nissl's method, the simplest form of which, as given by Guyer, uses 3.75 g. of methylen blue, 1.75 g. of venetian soap (white castile soap) dissolved in 1,000 cc. of water. Keep the stain for several months before using; then heat a small amount in a test tube until it steams. Use medium-sized pieces of spinal ganglion fixed for 3 days in 95A, then dehydrated, cleared, and

imbedded in paraffin, sectioned, and the sections affixed to slides. Run sections down to water and apply the warm stain for 6 min. to a slide placed flat on the desk. Pour off the surplus and rinse in distilled water. Lay the slide flat again and flood with anilin-alcohol (9 parts 95A, 1 part anilin oil) to decolorize, 20 to 30 sec., until pale blue. Drain and place the section in 100A, clear in xylene, and mount in balsam. The Nissl granules should appear deep blue.

*Golgi's chrom-silver method* for blackening nerve cells is a classic preparation but never has been made certain, the results being more or less unpredictable and only a proportion of slides or trials turning out successfully. Guyer advises the following modification of Golgi's rapid method: Fix small pieces of spinal cord in at least ten times their volume of 10% formalin for not less than 3 days, a longer stay doing no harm. When ready to proceed, again cut each piece into two or more smaller ones and place several in twenty times their volume of a 3.5 aqueous solution of potassium bichromate for 2 days, changing the fluid after the first day. Next impregnate with a silver nitrate solution made by dissolving 1.5 g. of silver nitrate crystals in 200 cc. of distilled water plus 1 drop of concentrated formic acid. Use a small quantity of this impregnator and rock the vessel gently until the brown precipitate of silver chromate ceases to appear.

Now transfer the pieces to forty times their bulk of fresh impregnator and leave for 3 days in the dark, changing the solution after the first day. Place pieces in 95A for 30 min., changing the alcohol after 10 and again after 20 min. Leave the remaining pieces in the impregnator for use in case the first lot proves a failure. Transfer the pieces being processed to 100A for 20 min., changing the alcohol after 10 min., then into ether-alcohol, 20 min. Imbed in thin celloidin, without infiltration, 30 min., and change to thick celloidin, 10 min. Mount on a block and harden in chloroform, 20 min.; thence to cedar oil until

clear, 30 to 60 min., and cut sections by the celloidin method, 50 to 100 microns in thickness. Keep the knife flooded with cedar oil and not with alcohol. Place sections in a watch glass of cedar oil until completely cleared, then place on a slide flooded with the cedar oil and inspect under the microscope, selecting good ones and throwing away the remainder. Replace oil with xylene for a few minutes, then drain and apply blotting paper gently to remove xylene. Cover a chosen section on the slide with thick balsam. It is important to note that no cover glass is put on a Golgi slide. The section must have access to air for evaporation of moisture, otherwise the silver deposits break up and ruin the preparation. Put the slides away flat in a dust-free place until the balsam is thoroughly hardened. By using the clarite technique explained in the preceding chapter, Golgi preparations may be mounted under cover glasses.

Other structures have also yielded excellent results by this technique, including bile capillaries in sections of the liver, the finer ducts of such glands as the pancreas or salivary glands, and the tracheae of insects.

*Cajal's method for neurofibrils* is a silver-reduction photographic process of great importance in neurology. Small pieces of nervous tissues, such as brain, spinal cord, or peripheral nerves, endings, or ganglia, are fixed in 15% formalin for 6 to 12 hr., then washed for 6 hr. in running tap water. Place in 50 cc. of 50A to which 5 drops of ammonia have been added, 24 hr. Wipe with blotting paper and impregnate with a 1.5% solution of silver nitrate in an incubator at 38° C. for 5 days. The silver is now reduced by bathing the tissue for 24 hr. in 100 cc. of distilled water, 10 cc. of formalin and 1 g. of pyrogallol or hydroquinone (hydroquinone). Rinse in distilled water, dehydrate, and continue with the usual paraffin or celloidin steps.

*Gold Chloride Method for Motor Nerve Endings.*—Kill a snake by decapitation or a blow on the head with a hammer

and open it up with a mid-ventral incision after its reflex actions have ceased. Using a seeker and hand lens, find one of the spinal nerves coming from the mid-dorsal region of the body cavity and running to one of the intercostal muscles, which are situated between the ribs. With fine scissors, remove a small piece of such muscle with a bit of the nerve attached and fix for 24 hr. or longer in 10% formalin. Transfer to 10% formic acid in distilled water, 40 min., then to a 1% solution of gold chloride in distilled water for 40 min. more. Although it is not necessary to impregnate with the gold in darkness, one should avoid direct sunlight. Reduce in the dark in 2% formic acid for 24 to 48 hr., examining occasionally to observe the color of the muscle fibers. They are yellow at first and gradually change through red to violet and then blue. Reddish-violet fibers are correct; blue ones have gone too far. When seen to be reddish violet, wash the material in several changes of distilled water for 1 hr., then place on a slide and tease with dissecting needles in distilled water. The object is to spread out striated muscle fibers so that separate elements are clearly seen, together with the jet-black nerve fibers and their end organs or motor plates. Cut away excess materials, but be careful at all times not to tear the very delicate and fine nerve endings from the muscle fibers. Dehydrate, clear, and mount in balsam. Some technicians prefer to immerse and surround the tissues with glycerin, then surround the glycerin with balsam, and add a cover glass. Parts of such slides have a messy appearance when superficially observed; they are excellent under high power, nevertheless, and will keep for long periods.

**Frog Eggs and Tadpoles.**—Whole mounts of all stages of frog development, from the one-celled egg to small tadpoles may be made in a variety of ways, from thick balsam mounts on plain slides, using glass strips as cover props, to cell mounts, or by the use of various depression and deep-well slides. Some workers prefer to mount each stage

on a separate slide; others use one slide for a series on a single subject, as cleavage, gastrulation, embryo formation; still others make a composite slide with one example of each stage on a single mount.

Sections run all the way from bisected eggs and larvae, through single sections to serial sections. One very instructive method is to mount one example of the entire object—for instance, a late cleavage stage—together with a bisected example of the same stage, cut surface uppermost, on the same slide. This gives an external and an internal view together. Some general hints follow.

Almost any species of frog or salamander will serve, though the leopard frog is most popular because of its great abundance, proper size, and ease of collection. The somewhat larger eggs of the spotted salamander, *Ambystoma maculatum*, are even better when obtainable, because of their lighter color. These animals emerge from hibernation at an early date, depending upon the latitude in which they live, and immediately repair to the ponds for breeding, which is always performed in the water. Often the ice is not entirely gone from the breeding pools when egg laying begins. One must learn the correct time for collecting according to locality (it will generally vary between the last two weeks in March and the first two in April), and should further consult the game laws of the particular state, since the taking of eggs is prohibited in some areas, and permission to secure a small amount for scientific purposes must be obtained from the game warden.

Collect early in the morning, at or just before sunrise, since it is then the clusters are laid and one must secure a freshly laid mass if the one-celled (uncleaved) stage is to be obtained. Bring an egg mass back to your laboratory in a bucket of the pond water in which they were collected and proceed to fix one or a few uncleaved eggs at once. From time to time, inspect sample eggs from the mass with a hand lens or dissecting microscope and in this way find the 2-, 4-, 8-celled, etc., stages of cleavage, a few of



which are then fixed; continue to remove examples as the various stages of development appear. Keep the cluster meanwhile in an aquarium or gallon battery jar of pond water, covered with a glass plate, on a window ledge or near by. Direct sunlight for too long a period continuously will overheat the water and probably kill the eggs.

As collected, each egg is surrounded by a gelatinous coat which must be removed. Two chief methods are recommended for this purpose: adhesion and digestion. By the adhesion method, transfer an egg to a blotter and very gently roll the egg toward the edge of it, using a camel's-hair brush or toothpick. The trick is to have the egg arrive at the edge just as the last bit of jelly has been rolled off by adhesion to the blotter, and then to poke the egg over the edge into a vial of fixer; or roll it along the edge until this is accomplished and then into the vial. A very little practice makes one adept at this work. For preparation of eggs in any quantity, however, use the digestion method, which consists in first fixing, then placing the fixed eggs in the digester for a few minutes until, by shaking, the eggs become free from the jelly. A stock solution is made, 10% sodium hypochlorite in water; to use this, it is diluted. Take 1 part of the stock and 5 parts of water; follow by rinsing in 35A.

To handle eggs during various manipulations from one reagent to another, nothing is better than a pipette made by substituting a short length of glass tubing for the constricted glass barrel of the pipette, forming a wide-mouthed pipette or dipping tube. Some prefer to cut off the narrowed portion of a medicine dropper, anneal the cut end, and slip over this a short length (1 in.) of rubber tubing, half of which projects as the working end of the pipette. Such implements have a sufficient caliber and will not injure delicate objects. Syracuse watch glasses and shell vials are the most serviceable forms of glassware for fixing, dehydration, staining, and storing.

For whole mounts, fix for overnight or 24 hr. in 10%

formalin, followed by washing in 70A for 24 hr., and 82A for 24 hr. Dehydrate and clear in the usual manner. Still better preparations can be made if, after the fixing and alcohol washing, the specimens are placed in a vial of hydrogen peroxide for about a week, until bleached a light brown, since the dark pigment of frog eggs interferes with observation of the cleavage furrows and, with tadpoles, of numerous external features. Follow the bleaching by dehydration and clearing.

For sections, fix in Tellyesnický's fluid, modified by the addition of 10 cc. of formalin to each 100 cc. of Tellyesnický. Fix for 24 hr., wash in running water, 6 hr., and dehydrate more gradually than usual, beginning with a 15% alcohol, thence proceeding through 25A and then the regular series. The difficulties attending the sectioning of frog eggs, which are extremely brittle, have already been discussed and either celloidin, Johnson's asphalt-paraffin, or Hance's rubber-paraffin-beeswax method is recommended. Thick sections are frequently better than thin ones in this study. Sections of later stages may be cut in any of the three planes used in embryology; cross (transverse), sagittal (median longitudinal, passing through back and belly), or frontal (longitudinal, passing through right and left sides). Strictly avoid all oblique sections, not in one of these three planes. Serial sections are discussed under the next topic, on chick embryos.

To bisect an egg or larva, nothing is better than a fresh safety-razor blade, with or without a holder. Make a clean cut in a definite, predetermined plane. This provides material for two mounts, if more than one is desired.

Staining is optional, many workers preferring bleached but unstained material. If a stain is used, apply it lightly; tinge but do not dye heavily. If overstained, use an acid destain to lighten the color. Alum cochineal and borax carmine are widely used general stains, especially for tadpoles; diluted fast green makes a very pretty preparation for bleached eggs. Cedar oil is a better clearer than xylene,

though the latter will serve. Among the newer techniques, the *n*-butyl alcohol method is highly recommended.

In mounting entire specimens or bisected stages on plain slides, one stage per slide, it is best to use the spot method. With a toothpick, place a very small drop of airplane cement on the slide at the place where the mount is to be made; then transfer the cleared egg or larva to this drop and orient it so that the view wanted is uppermost. Allow it to set for a moment but not long enough for the specimen itself to dry out, then add thick balsam, props, and a cover, carefully, trying not to disturb the position. Keep perfectly flat until the balsam has hardened, preferably hastening the process in a slide drier. Heat the cover glass before applying and press it down, but avoid inclusion of air bubbles.

A most instructive preparation is made with a deep-well slide. Select one perfect specimen of each of some 10 stages: 1-, 2-, 4-, 8-, 16-celled stages of cleavage, many-celled or blastula, crescent blastopore or yolk plug, neural groove or tube, tail bud, and tailed larva. When these have been fixed and bleached, with or without staining as desired, they are placed in 10% formalin in the well of the slide, filled flush with the fluid. A circular cover glass larger than the opening of the well is placed on and sealed with several coats of asphalt varnish or lacquer. The only trick is to avoid, as much as possible, inclusion of an objectionably large air bubble, by having the well absolutely full. A small air bubble will generally develop, at first or later, which will not affect the preparation or destroy its utility. By tilting the slide, specimens may be made to tumble about in the formalin so as to present different aspects for low power or binocular examination.

All whole mounts and bisections should be studied by reflected as well as by transmitted light.

**Chick Embryos.**—This phase of microtechnique is fascinating and may well detain the amateur for a number of

months; in fact, the whole field of embryology is one of the best specialties that could be selected as far as interest and importance are concerned. The chick has become a classic and all microscopists should familiarize themselves with at least the primary technique of preparing whole mounts and sections of various ages.

The first essential is a source for fertile eggs of known age. If one lives in the city, contact with some farm on which numbers of eggs are regularly set should be established, either direct or through a grocer. The second requirement is an incubator capable of being accurately regulated within  $1^{\circ}\text{C}$ ., since eggs must be incubated at  $39^{\circ}\text{C}$ . ( $102^{\circ}\text{F}$ .) for development to proceed; a temperature higher than  $40^{\circ}\text{C}$ . will kill the embryo. The ages of the eggs should be written on the shells, and are calculated in hours of incubation, either by a setting hen or an incubator. Development ceases when the eggs are cooled, but they remain alive and will resume development when again warmed. Obtain eggs a few at a time, of known hours of incubation.

The standard ages for embryology are 18, 24, 33, 48, and 72 hr. A more complete series would add stages of 12, 56, 64, 84, and 96 hr. of incubation. Choice of these hours is not solely arbitrary. Since certain stages in the development of definite organs are completed or have reached a typical condition by the times stated, such material is most instructive for study. A number of eggs opened are likely to prove sterile, hence a few more than wanted of each stage should be at hand to allow for such failures.

Eggs should be handled carefully and placed on shelves of the incubator in rows according to age desired. Maintain the temperature accurately, see that any fumes, as from a gas flame, are carried away, and have a vessel of water present to moisten the air. In keeping eggs thus incubated for the later stages, turn them every 2 days, just as the hen would do. Complete development requires 21 days; if an entire series of 21 specimens is desired, the older chicks, above 96 hr., are preserved as entire objects in vials and jars.

It is suggested that slide work begin with a whole mount of a 48-hr. chick. Remove such an egg from the incubator, break the shell of the blunt end where the air chamber is situated, and place the egg in a vessel of physiological salt solution, warmed to 38 to 40° C. A biological finger bowl is good for this purpose, though any glass mixing bowl or china vegetable dish serves equally well as long as it is deep enough to immerse the egg completely and allow room for the hands. With forceps, remove pieces of the shell and shell membrane, bit by bit, working from the cracked shell at the blunt end to the center of the top as the egg lies in place. Do not change the position of the egg during shell removal. That part of the ball of yolk which bears the embryo has a lighter specific gravity and will float uppermost, turning slowly upon two specialized and twisted strands of albumen that serve as axes. After an opening in the top center some 1½ in. in diameter has been made, the embryo may be seen as a circular disk lying upon the yolk. This disk is termed the "blastoderm," "blastodisk," or "germinal disk," and by the 48-hr. stage has attained considerable size.

During shell removal, if any of the white (albumen) tends to flow out, cut it off immediately with sharp scissors at the shell margin, otherwise it will drag on the yolk and probably roll the blastoderm over out of sight. With a pipette, remove the albumen lying over the blastoderm, thus exposing the embryo fully to view. The heart will be seen beating at this age and, if the egg is kept in warm salt solution, the chick will remain alive for a considerable period and can be observed as desired under magnification. From this point on, there are two schools of technique. In one the blastoderm is fixed while still in place; in the other it is first removed and then fixed. There is little to choose from between the two and it is advised that the beginner try first one and then the other, as different ages of whole mounts are prepared.

*Method 1:* Flood the exposed embryo with the fixer,

dropping it on slowly and gently with a pipette. For this purpose, Bouin's fluid, corrosive-acetic, or other good general fixer may be used. However, in order to introduce a new reagent, we shall here recommend one that has yielded most excellent results with chick embryos, Kleinenberg's picric-sulphuric. As soon as the blastoderm has acquired a completely opaque appearance, cut around it rapidly with a fine scissors—curved blades are most useful—and make the cut just inside the region where the blastoderm joins the ball of yolk. Grasp the edge of the blastoderm with a fine forceps and shake it back and forth very gently to free it from the underlying yolk, then swim it into a watch glass which has meanwhile been immersed in the salt solution, beside the egg, or work it onto a slide similarly held beneath the solution.

Andrews applies the fixer by a careful and excellent method, using a curved pipette with the end drawn out in a flame to a fine point. There is a very delicate membrane surrounding the entire yolk and enclosing the embryo—the vitelline membrane—and the first injection of fixer is made between it and the embryo, at the edge of the blastoderm. A second injection is then made between the blastoderm and the yolk. The blastoderm is then cut loose and removed as before.

After removal, the blastoderm being in a watch glass, the vitelline membrane is cautiously removed and the salt solution and any adhering yolk granules are sucked up with a pipette to the point of complete removal, keeping the specimen flat meanwhile, and the fixer then carefully pipetted or poured on to cover the specimen, which remains in the fixer for 3 hr. An excellent procedure, recommended by many operatives, is to cut a piece of filter paper the same size as the blastoderm, then to cut out a circular hole in the center of this piece so that it is equal in size to the embryonic area—the part which is to be mounted—including the embryo itself and the opaque area immediately surrounding. This ring of paper is placed over the embryo as

it lies in the empty watch glass or on a slide, before the fixer is added. It will adhere to the outer parts of the blastoderm, prevent curling of edges, and provide the worker with a firmer support to grasp with forceps during subsequent manipulations. Still others use a circular piece of filter paper without the central hole, placing this over the embryo so that the outer edge catches and holds the outer membranes. This also prevents curling and provides a firmer support for handling. Shumway works a square cover glass under the blastoderm and uses this as a support.

*Method 2:* Cut out the unfixed blastoderm as heretofore directed and wave it gently in the salt solution to remove yolk. Swim the blastoderm into a syracuse watch glass or onto a slide and with fine forceps and needles remove the vitelline membrane. Support and flatten the specimen by one of the methods already enumerated, while situated in a second and dry watch glass; then add the fixer with a pipette. When fully opaque, add more fixer until the chick is well covered, and allow to act for 3 hr.

By either method, it is essential to know at all times which surface of the blastoderm is uppermost. Slides are generally prepared with the dorsal surface up, as the specimen lies in the egg; if a full series is desired, it is well to have two of each age, the second of each with the ventral surface up.

Following fixation, wash in a number of changes of 70A for several hours, then pass through 50A and 35A, 1 hr. each, to water. Stain for 3 hr. in Conklin's picro-hematoxylin, wash in water, then dehydrate for 30 min. each in 35 and 50A. Destain to the desired degree in 70% acid alcohol, restore the blue color with alkaline alcohol, dehydrate through 82, 95, and 100A, 30 min. each, and clear in half 100A, half xylene, 30 min., then in pure xylene until clear and transparent, the average time being about 2 hr. Cedarwood oil is preferred by many; McClung and Allen state that synthetic oil of cassia (cinnamic aldehyde) is much the best clearer for chick embryos since it causes the

smallest amount of shrinkage. Note also our previous mention of the *n*-butyl alcohol method for embryos.

When the specimen is clear, trim any ragged edges with scissors and mount in balsam, with cover glass props. Remove any supporting paper or cover glass before mounting.

In general, the pipette-fixation method of Andrews is best for early stages (12 to 24 hr.), the procedure here termed method 1 for intermediate stages (33 to 48 hr.), method 2 for stages up to 84 hr.; from 96 hr. onward, embryos are merely clipped out and dropped directly in the fixing fluid. Avoid handling the material as much as possible. Make changes of reagents by pipetting the old one out and the new one in as the blastoderm lies in the watch glass.

Preparation for sectioning does not differ from ordinary histological procedure except that the delicacy of the material necessitates greater than usual care in handling. Imbed in a medium-soft paraffin or a lanolin-paraffin for 3 hr., and cut sections 20 to 30 microns in thickness on a rotary microtome. While still cutting paraffin alone, before reaching the level of the embryo, see that the paraffin is ribboning nicely, evenly, and without holes, cracks, splits, or other defects. It is very necessary to orient the embryo accurately in the paraffin block and to know, when the block is mounted on the object carrier of the microtome, which way the embryo lies as regards right and left sides, anterior and posterior ends, dorsal and ventral surfaces. Have a number of albumenized slides prepared in advance.

The paraffin block is affixed to the object carrier, and the latter so adjusted in the clamp that cross sections will be cut, beginning with the head end and ending with the tail, the knife entering the left side of the chick's body first. In sagittal sections, the right side of the body is cut first, the knife entering the ventral surface; with frontal sections, the ventral surface is cut first and the knife enters the left side. Trim the paraffin blocks so that the sections in the ribbon will be only about 4 mm. apart, that is, with a mar-



gin of about 2 mm. beyond the embryo on all sides of the block.

In mounting serial cross sections, which are by far the most useful of the three planes of sectioning, allow room for the slide label on the left; then begin in the upper left corner of the remaining space and lay out the ribbon from left to right, like printed matter on the pages of a book, along the upper portion of the slide, fairly near the edge but allowing room of course for balsam and the cover glass to enclose all parts completely. The dorsal surface should be down so that it appears up through the microscope. Cut the ribbon near the right edge of the slide and begin again with a second row, underneath the first, and continue in this fashion for four or more rows, ending at the bottom right corner. An 18- or 24-hr. chick should be mounted complete on a single slide; with the skill resulting from practice, some technicians can get even a 48-hr. chick on one slide. More often, however, from 33 hr. up, additional slides are needed, until a total of nine, ten, or more slides may be required for a 72-hr. or older chick, since probably no more than two rows per slide can be made with these older stages.

After finishing the first slide, resume operations with the remaining ribbon on a second slide, exactly as before. When finally completed, such slides are labeled so as to indicate their position in the series, for example, "Chick Embryo/48 hr. serial c. s./*A* - 1." The second slide bears the same data except that the last line is "*A* - 2"; the third slide "*A* - 3," and so on. *A* indicates the series and avoids mixing where more than one series of the same age are made. The next series would be labeled "*B* - 1," "*B* - 2," and so on. This is especially necessary in teaching collections, where many series of the same age may be kept on hand.

In order to be able at all times to distinguish surfaces during cutting and mounting, many technicians prefer to trim the paraffin block unsymmetrically, cutting one of the vertical sides square, the other oblique, or both oblique,

so that the edge of the block entered by the knife is longer than the opposite face. In all cases, however, that edge must be strictly parallel to the opposite edge; only the sides may be slanted.

As paraffin sections are affixed to slides, be sure to put on a temporary label or mark with a glass pencil so that slides do not become mixed. Some prefer to stain the entire embryo (staining in bulk) before sectioning, in which case borax carmine is very serviceable. However, much better and more precise results are obtained by staining the sections on slides, as with any other tissue. For this purpose, again, borax carmine or alum cochineal may be used as a single stain; double staining in the way given for ordinary tissues is much better. Delafield's hematoxylin and eosin make a good combination, also borax carmine and Lyons blue or fast green. Iron hematoxylin with a light counterstain, as orange G, makes beautiful slides that will well repay the extra trouble and are especially desirable for any detailed cellular work.

As an example of one of the more advanced staining techniques with serial sections of chick or pig embryos, try the fuchsin-picro-indigo-carmine method. With a slide of sections run down to water, stain for 20 min. in a saturated aqueous solution of basic fuchsin, rinse in distilled water, and counterstain for 5 min. in picro-indigo-carmine, which is a mixture of equal parts of saturated aqueous solutions of picric acid and of indigo-carmine. Dehydrate rapidly by passing through 70, 95, and 100A, then into 100A plus xylene (equal parts), then xylene, and mount in balsam. The 70A takes out the green; the 100A extracts red. Only experience will teach the operator the right duration in each alcohol while dehydrating in order to get the best results.

Large-sized rectangular cover glasses are used for serial sections, the preliminary cleaning of which is more difficult than for smaller sizes, owing to the ease with which they are broken. One of the best methods is, after washing in one of

the cleaning fluids and rinsing in water, to cover two small blocks of perfectly flat and smooth wood with clean linen, as from an old handkerchief, and polish the cover between these blocks. Handle it thereafter with a forceps and flame it gingerly before applying. See that it trues up with the slide and covers all sections before placing the slide away to dry.

**Pig Embryos**, the most widely used examples for mammalian development, may be obtained from supply houses or easily procured personally if a slaughterhouse exists in your vicinity. The two horns of the uterus (uterine tubes) of a slaughtered female pig are slit lengthwise, exposing embryos as they lie in their membranes, which are then removed intact and placed in the fixing fluid. Afterward the membranes are opened to secure the embryos proper. Stages of 6 and 10 mm. (crown-rump measurement) are desired for whole mounts and sections; older embryos may be sectioned or put up whole as preserved specimens in vials and jars. The procedures are the same as for chick embryos as regards fixation, washing, staining, and so on, for either whole mounts or serial sections.

At the same time that pig embryos are secured at an abattoir, advantage should be taken of the opportunity to secure a small piece of placenta for sectioning, and of the liver, which has a more primitive structure in the pig than in most other mammals, and of any other tissues or organs desired, as well as a search of the digestive tract for helminth parasites.

**Injections.**—Blood vessels of embryos or adults of higher animals may be injected with colored solutions in order to make them easier to follow during an anatomical dissection or to show up the relationships of the blood supply to various sectioned organs in histology. For gross dissections, the injection masses may consist of gelatin, cornstarch, or latex, suitably tinted with brilliant red or blue dyes, the formulas

for which appear in the next chapter. Injections may be further designated as single, double, or even triple, according to the kind of vessels involved. Single injections display the entire arterial system; double ones color arteries one hue and veins another; triple are used when some special system, as the hepatic portal veins are given a third color. As an example, let us say that we are going to make a single injection in a cat to obtain microscope slide cross sections of the small intestine which, in addition to revealing the usual histological construction, will show also the arteries supplying all parts—indeed, a beautiful and instructive slide.

Kill a cat and work rapidly so as to complete the injection while the animal is still warm. Open up the abdominal cavity and expose the coils of the small intestine, along with the major arteries that arise from the dorsal aorta (main longitudinal arterial trunk running along the extreme mid-dorsal wall of the body cavity) and course through the mesentery (supporting membrane of the intestine). Fill a small metal or glass syringe with a red injection mass and insert the tip of the needle in one of these arteries, toward the intestine. Work a thread under this vessel and tie a loose knot around the vessel and the inserted needle so that the artery may be tied off when the injection is completed. Inject with a slow, steady, and light pressure, tie the knot tightly, then remove the needle.

Immerse the cat in cold water for 30 min., then remove a small section of the injected intestine, as for regular fixation, and harden in 10% formalin for 24 hr. Regular technique then follows; the paraffin method is not satisfactory as a rule owing to the necessity for the employment of heat. If you have the necessary equipment, use the freezing or celloidin routines; if not, make sections free hand or in a well microtome, a quite satisfactory substitute, since they should be thick (30 to 50 microns) in any case. Unstained sections are very good, but try staining at least some with hematoxylin and light green, neither of which colors will interfere with the red of the arteries.

If you wish a number of injected organs, a single cat will provide as many as desired but the injection should be made into the left ventricle of the heart so as to fill the entire body at one time. The proper dissection to make for this purpose is to cut through the skin in the mid-line of the belly surface, from the abdomen to the neck, then to cut to each side at each end of this incision so that flaps of the skin may be laid back out of the way. Rapidly cut through the cartilaginous parts of the ribs of each side with a blunt and fairly heavy scissors, up to the level of the first rib. Turn the sternum (breastbone) forward and tie it off with strong thread opposite the first pair of ribs so that bleeding from cut vessels will be prevented. Expose the heart and snip off the posterior tip with scissors so that the cavities of the two ventricles may be seen. Sponge away the blood flowing from them, then insert the hypodermic needle into the left ventricle (the cat's left, not the operator's) and on through to the aorta, the great artery that arises from this ventricle. Prepare a thread to tie off this aorta; then proceed to inject.

The needle should be of large diameter and the ligature (thread) tied tightly around the aorta with its included needle, to prevent backflow. Use a light, steady pressure, which must be increased as the injection proceeds, but is never strong enough to rupture small vessels. Examine the intestine and gums from time to time to note progress of the injection mass and when to stop. When finished, remove small pieces of as many organs as desired and treat as previously directed for the intestine.

If needles of proper sizes are not available, make a glass canula by drawing out glass tubing of suitable diameter in a flame and connect it to the syringe with rubber tubing.

For a double injection, use first a blue gelatin mass and inject until it is seen to issue from the right ventricle. Disconnect the syringe from the needle or canula, leaving the latter in place, and clean quickly and thoroughly with warm water; then fill it with a red cornstarch injection mass and reattach to canula. Inject until the red shows up in

small vessels in several areas exposed for control, as a thigh muscle, intestine, or gums. The red drives the blue ahead of it into most of the veins and will not reach them itself since the particle size of the cornstarch mass is too great to pass through the capillaries. Hence the veins of organs such as the kidneys will be filled with a blue mass and the arteries with red. The larger veins of the arms and legs are provided with valves to prevent backflow and these cannot be filled; but that is of no importance in the usual histological requirements where only small bits of organs are needed. This double injection will not fill the veins draining the digestive tract and spleen. For these a separate injection is necessary, inserting the canula in the portal vein where it enters the liver and injecting toward the rear.

For chick embryos, india ink is almost universally employed as the injection mass and a glass canula must be prepared. Heat a short (4 to 6 in.) length of small-diameter, soft glass tubing over a moderate bunsen burner flame, rotating the tube constantly as it is held between the thumb and forefinger of each hand and directing the flame against the middle of the tube alone. When this spot is soft, remove from flame and quickly pull and bend simultaneously so that the tube is both drawn out and bent to a right angle. Using a file, break the tube at the apex of this angle, giving two canulas with curved tips.

If the opening at the tip is either too large or closed completely, as is apt to be the case, reheat and draw out the tip with a fine forceps. Slip one end of a 16 to 20 in. length of rubber tubing of appropriate diameter over the larger end of this glass canula and by suction with the mouth, fill the glass tube with india ink.

Before filling tube with ink, prepare a chick embryo of 48-hr. age or up by removing the shell and floating the yolk in a vessel of normal saline at 39 to 40° C. Observe the beating heart and circulating blood; there is a vessel running in circular fashion almost completely around the blastoderm, termed the "marginal vein"; the prominent trunks running

from this to the chick proper are vitelline veins. The best site for injection is the marginal vein near the point of departure of the vitellines. Holding the freshly filled injection canula in the right hand, just above and in line with the marginal vein, the tip end toward the junction of the vitelline vein, make a gentle downward movement so that the tip of the canula will pierce the marginal vein, an operation not quite so difficult as it sounds. Blow very gently through the rubber tubing, causing the ink to be injected. Watch closely and, as soon as the stream of black ink reaches the heart, discontinue blowing. The beating heart will now continue to work for you and will pump ink throughout the circulatory system, filling all vessels. Remove the canula when the injection has been thus completed and proceed with regular fixation of the blastoderm. Mount with the dorsal surface up, unstained.

## CHAPTER 17

### PREPARATION AND USE OF REAGENTS

*In This Chapter:* formulas for the manufacture and rules for use of all general reagents mentioned in this book, together with a few others of standard usage. Unusual formulas for very special cases, already given in the body of the book, are not repeated here.

#### GLASS-CLEANING MIXTURES

**Acid-Alcohol Mixture.**—Equal parts, hydrochloric acid and 95A.

**Bichromate-Sulphuric Mixture.**—Most powerful of all cleaning media.

Potassium bichromate.....	10 g.
Sulphuric acid, concentrated, commercial.....	40 cc.
Water, tap.....	50 cc.

Dissolve bichromate crystals in the water by heating, then cool. Add the acid a little at a time and very cautiously, as the reaction generates a terrific heat. Never add the water to the acid; always the acid to the water. May be used full strength or diluted.

*With Both of the Foregoing:* Leave glassware in cleaner overnight or 24 hr., then rinse in tap water, and remove to a jar of strong hot soapsuds, for overnight or 24 hr. Rinse and either dry at once or place in alcohol until needed. Do not drain these strong acids down the sink; do not immerse the hands in them. Cleaners may be used repeatedly.

**Metasilicate Cleaner.**—For earthenware, porcelain, and metal containers as well as glass.

Sodium metasilicate.....	3 g.
Water, tap, hot.....	97 cc.



**Slides and Covers** with balsam, as old or rejected mounts.

Warm gently first to melt the balsam, then place in any of the foregoing cleaners. It is best to push covers off slowly with a matchstick into one container, then put slides in a second one, keeping them separate.

**Bon Ami Cleaner** has never been surpassed for polishing slides and covers. After preliminary cleaning in any of the above, or in water, or waste alcohol, place in the following emulsion:

Bon Ami powder.....	5 g.
Water, tap, hot.....	100 cc.

After soaking in above, remove slides to drain, standing them against a vertical support or in trays lined with cheesecloth. When dry, clean at once, or leave with powder on them and clean as needed.

### KILLING AND FIXING AGENTS

Classified under principal ingredient, arranged alphabetically; cross-referenced to authors. Fixers are used only once.

#### **Alcohol.**

**ABSOLUTE ALCOHOL.** Generally considered inferior for fixing when used alone.

*Indicated:* when nothing else is available; nervous tissue for Nissl's method; aorta for elastic tissue; boiling, for insects. Use large quantities. Very easy to use since there is no washing; pass directly to imbedding or staining.

*Time:* 1 hr. to 1 day according to size and density of object. Change once or twice.

*Stains:* nearly any stain may follow; hematoxylin, acid fuchsin.

#### **ALCOHOL-ACETIC (Carnoy's Fluid, No. 1).**

Absolute alcohol.....	3 parts
Acetic acid, glacial.....	1 part

*Indicated:* much preferable to alcohol alone, the acetic counteracting shrinkage caused by the alcohol; penetration, very rapid; use, general; has been very successful with the difficult ascaris eggs.

*Time:* 30 min. to 1 hr.

*Wash:* 100A, several changes, until odor of acetic acid disappears.

*Stains:* any.

#### ALCOHOL-ACETIC-CHLOROFORM (Carnoy's Fluid, No. 2).

Absolute alcohol.....	6 parts
Chlcroform.....	3 parts
Acetic acid, glacial.....	1 part

*Indicated:* general use; especially lymphatic and glandular tissues, onion root tips.

*Time:* even more rapid than the preceding, the chloroform hastening the process; 10 min. to 1 hr.

*Wash:* same as preceding; 100A.

*Stains:* any; the following combinations are notably brilliant after this fixer: cyanin and erythrosin, acid fuchsin and iodine green.

#### ALCOHOL-ACETIC-CHLOROFORM-CORROSIVE (Carnoy and Lebrun).

*Making:* mix equal parts of absolute alcohol, chloroform, and glacial acetic acid, then saturate this mixture with corrosive sublimate.

*Indicated:* best of all alcohol mixtures for fine cytological work; suitable for all vertebrate material.

*Time:* 10 to 30 min.

*Wash:* 100A, colored with iodine solution. Fresh 100A.

*Stains:* any combination.

**Bichloride of Mercury:** see Mercuric Chloride.

**Bichromate of Potassium:** see Potassium Bichromate.

**Bouin's Fluid:** see Picric-Acetic-Formalin.

**Carnoy's Fluid:** see the several Alcohol-Acetic formulas.

**Champy's Mixture:** see Chrom-Osmic-Bichromate.

**Chromic Acid.**

**CHROMIC ACID.** As is usually the case, is better in mixtures than alone. Is one of the best hardeners known, but makes tissues brittle. Essential to use large quantities, *e.g.* 200 cc. for a piece of tissue 1 cc.; change to fresh at least once.

*Indicated:* primarily for botanical material; also for cytology, as nervous tissue, ovary. Usually employed as 1% aqueous solution.

*Time:* fixing, 24 to 48 hr.; hardening, several days to 6 wk. or longer.

*Wash:* running water, 24 to 48 hr., in the dark.

*Stains:* hematoxylin or basic anilin dyes.

**CHROM-ACETIC ACID.**

Chromic acid.....	1 g.
Acetic acid, glacial.....	1 cc.
Water, distilled.....	100 cc.

*Making:* this is a stock solution from which a great variety of strengths can be made, every author having different preferences. Thus if 50 cc. of the stock and 50 cc. water are mixed, each acid is cut to half strength; then if to this 2.5 cc. glacial acetic are added, the final mixture is one of 0.5% chromic and 3% acetic. Chamberlain recommends a weak solution (chromic, 0.3 g.; acetic, 0.7 cc.; water, 99 cc.) and a strong one (chromic, 1 g.; acetic, 3 cc.; water, 100 cc.).

*Indicated:* primarily botanical, the stock or weak solutions for embryos, the strong for thallophytes and bryozoa; also for earthworms.

*Time:* 24 to 48 hr.

*Wash:* running water, 24 hr.

*Stains:* hematoxylin or basic anilin dyes.

**CHROM-ACETIC-BICHROMATE (Goldsmith's Mixture).**

Chromic acid, 1% aqueous solution.....	15 parts
Potassium bichromate, 2% aqueous solution.....	4 parts
Acetic acid, glacial.....	1 part

*Indicated:* protozoa, planaria.

*Time:* 2 to 24 hr.

*Wash:* 6 to 12 hr., running water.

*Stains:* as in preceding; iron hematoxylin follows well.

#### CHROM-ACETIC-FORMALIN.

Chromic acid, 1% aqueous solution..... 80 cc.

Acetic acid, glacial..... 5 cc.

*Making:* the above is the stock solution. To use, Licent adds 15 cc. formalin to fix algae and fungi; Guyer takes 2 volumes of stock and 1 volume of formalin, mixing just before using, for general embryological work.

*Indicated:* thallophytes, embryology.

*Time:* 6 to 12 hr.

*Wash:* running water, 6 hr.

*Stains:* almost any stain will follow.

#### CHROM-ACETIC-OSMIC (Flemming Mixtures).

##### Strong Solution

Chromic acid, 1% aqueous solution..... 15 parts

Osmic acid, 2% aqueous solution..... 4 parts

Acetic acid, glacial..... 1 part

##### Weak Solution

Chromic acid, 1% aqueous solution..... 25 parts

Osmic acid, 1% aqueous solution..... 10 parts

Acetic acid, 1% aqueous solution..... 10 parts

Water, distilled..... 55 parts

*Making:* two procedures: (1) make fresh, immediately before using; (2) make chromic, acetic, and water as stock, then add the osmic immediately before using. The complete mixtures deteriorate on standing. See remarks on osmic acid, p. 297.

*Indicated:* primarily cytological, especially for mitosis and chromosomes. Also fat, epithelium, muscle, sense organs.

*Time:* 24 to 48 hr. Penetration slow; use only small pieces of tissue.

*Wash:* 6 to 24 hr., running water. Sections should be bleached on the slide before staining.

*Stains:* iron hematoxylin, safranin, gentian violet.

*Variation:*

Chicago Formula (Chamberlain)

Chromic acid.....	1 g.
Acetic acid, glacial.....	2 cc.
Osmic acid, 1% aqueous solution.....	6 to 8 cc.
Water, distilled.....	90 cc.

Add the osmic just before using. Excellent for root-tip mitosis.

**CHROM-OSMIC-BICHROMATE** (Champy's Mixture).

Chromic acid, 1% aqueous solution.....	7 parts
Potassium bichromate, 3% aqueous solution.....	7 parts
Osmic acid, 2% aqueous solution.....	4 parts

*Making:* this fixer may be made up in complete form at any time, as it keeps well.

*Indicated:* general use in cytology.

*Time:* 6 to 24 hr.

*Wash:* running water for same time as fixed.

*Stains:* iron hematoxylin, Champy-Kull triple stain.

**Corrosive Sublimate:** see Mercuric Chloride.

**Flemming Mixtures:** see Chrom-Acetic-Osmic.

**Formalin.**

**FORMALIN.** See general remarks, pp. 141, 273.

*Making:* 10% is the strength commonly employed when used as a fixer. This means 10 volumes of water to 1 of full strength commercial formalin. Use sea water in making up for fixing marine organisms.

*Indicated:* a good general fixer for all classes of materials, but other reagents are usually preferred for fine cytological work. Preserves fat and myelin, hence especially good when these are to be retained; also when microchemical tests are to be made; widely employed for brain and spinal cord; much used with the freezing method of sectioning. An ex-

cellent hardener; tissues become tough but not brittle.

*Time:* overnight to 24 hr. For large objects, as brain, fix for several weeks or even months.

*Wash:* 50A, several changes.

*Stains:* any stain may follow.

#### FORMALIN-ACETIC-ALCOHOL (FAA).

Formalin, commercial.....	5 cc.
Acetic acid, glacial.....	5 cc.
Alcohol, 50%.....	90 cc.

*Making:* a large number of variations on this theme are favored by different technicians, some of which are given here.

*Indicated:* often called the universal fixer; good for all general cases, but not recommended for fine cytological work. Especially adaptable for field collecting, as it both fixes and preserves and material may be left in it for months. Will fix an amount equal to its own weight.

*Time:* fixing, 24 to 48 hr.; preservation, indefinite duration.

*Wash:* 50A, several changes.

*Stains:* any stain may follow.

*Variants:*

##### Lavdowsky's Mixture

Formalin, commercial.....	6 parts
Acetic acid, glacial.....	1 part
Alcohol, 95%.....	20 parts
Water, distilled.....	40 parts

The original FAA formula is preferred by botanists, Lavdowsky's by zoologists.

##### Kahle's Fluid

Formalin, commercial.....	6 parts
Acetic acid, glacial.....	1 part
Alcohol, 95%.....	15 parts
Water, distilled.....	30 parts

Recommended for arthropods, especially insect larvae.

## Universal Preservative (General Biological Supply House)

Formalin, commercial.....	6.5 cc.
Acetic acid, glacial.....	2.5 cc.
Alcohol, 50%.....	100.0 cc.

For general fixing as well as preservation of entire objects, including field collecting.

## FORMALIN-CORROSIVE (Worcester's Fluid).

*Making:* 1. Make up a 10% solution of commercial formalin.

2. Saturate this solution with corrosive sublimate.

3. To 9 parts of this mixture, add 1 part glacial acetic acid. Prepare fresh, just before using.

*Indicated:* nervous tissue, embryology, protozoa.

*Time:* 6 to 24 hr.

*Wash:* running water, 6 to 12 hr.

*Stains:* any desired stain may follow.

## FORMALIN-CORROSIVE-BICHROMATE (Zenker Formalin Mixtures).

*Stock Solution:* same as Zenker's fluid; see under Potassium Bichromate-Corrosive.

*Helly's Fluid.*

*Making:* prepare a Zenker's fluid stock solution. For use, instead of adding acetic acid, add formalin in the same amount and same manner, *i.e.*, substitute formalin for acetic acid. Mix just before using, as the complete fixer will not keep well.

*Indicated:* for tissues in which examination of the cytoplasm is the main aim. Excellent also for all vertebrate material.

*Time, Wash, Stains:* same as for Zenker's.

*Maximow's Fluid.*

*Making:* same as Helly's except that the amount of formalin is doubled.

*Indicated:* same as Helly's; also especially recommended for all the lymphoid organs—spleen,

lymph glands, bone marrow—when studying reticular tissue.

*Time, Wash, Stains:* same as for Zenker's.

**Gage's Fluid:** see Picric-Alcohol.

**Gilson's Fluid:** see Corrosive-Nitric under Mercuric Chloride.

**Goldsmith's Mixture:** see Chrom-Acetic-Bichromate.

**Helly's Fluid:** see Formalin-Corrosive-Bichromate.

**Hermann's Fluid:** see Platinic-Acetic-Osmic.

**Kahle's Fluid:** see Formalin-Acetic-Alcohol.

**Kleinenberg's Mixture:** see Picric-Sulphuric.

**Lavdowsky's Mixture:** see Formalin-Acetic-Alcohol.

**Maximow's Fluid:** see Formalin-Corrosive-Bichromate.

**Mercuric Chloride:** (Bichloride of Mercury; Corrosive Sublimate).

**CORROSIVE SUBLIMATE.**

*Making:* this salt is customarily kept in the form of a saturated solution, generally in distilled water; 6 g. of the sublimate will saturate 100 cc. water (6% solution). Some technicians prefer to make the solution in normal saline; others in alcohol, which will take up a 33% solution. May be used alone, though any of the following combinations are generally preferable.

*Indicated:* glands, epithelia, spinal cord.

*Time:* a few seconds or minutes for very small and delicate objects; 1 to 6 hr. for objects of intermediate size and density; 24 hr. for the larger and denser types. Unlike many other fixers, the time duration is more important here, as corrosive will easily over-fix. Objects turn opaque in this solution and, when they have done so throughout, fixation is complete and they should be removed.



*Wash:* aqueous solutions, running water; alcoholic solutions, 70% alcohol; 6 to 24 hr.

*Iodine treatment:* it is essential in nearly all fixers containing mercuric chloride to remove excess by treatment with iodinated alcohol, otherwise insoluble black "pins" will form in the tissue. Tincture of iodine may be used, though most workers prefer a 10% solution of iodine in 70% alcohol. Some treat the fixed tissue; others the sectioned material on slides. In the tissue method, after washing with running water, transfer to 50A for 3 hr., then 70A for 24 hr. (average times). Now place tissue in fresh 70A to which enough of the iodine solution has been added to give it a port-wine color. If this color is extracted, and as often as extracted, change tissue to fresh iodinated 70A. After 24 to 48 hr. the color will persist, then pass to 82A to wash out the iodine; change this alcohol as often as it is discolored. Then complete the dehydration.

*Caution:* do not use any metal objects in connection with reagents containing mercuric chloride; it corrodes metal badly. Employ horn, glass, porcelain, or wooden spoons, containers, or implements.

*Stains:* any stain may follow; carmines are particularly brilliant.

#### CORROSIVE-ACETIC.

Corrosive sublimate, saturated aqueous solution.....	95 parts
Acetic acid, glacial.....	5 parts

*Indicated:* marine animals, invertebrates in general, insects, invertebrate eggs, vertebrate embryos.

*Other data:* see corrosive sublimate.

#### CORROSIVE-ACETIC-ALCOHOL (Schaudinn's Fluid).

Corrosive sublimate, saturated aqueous solution.....	40 parts
Alcohol, 95%.....	20 parts
Acetic acid, glacial.....	3 parts

*Indicated:* protozoa, free living or parasitic, including fecal smears. Use cold or warm, the latter being more rapid. See p. 167 for an example.

*Time:* 10 to 30 min.

*Wash and Aftertreatment:* same as with corrosive sublimate.

*Stains:* any may be used, iron hematoxylin being a favorite.

#### CORROSIVE-NITRIC (Gilson's Fluid).

Corrosive sublimate.....	5 g.
Nitric acid, 80%.....	4 cc.
Acetic acid, glacial.....	1 cc.
Alcohol, 95%.....	15 cc.
Water, distilled.....	220 cc.

*Making:* allow the mixture to stand 3 days, then filter.

*Indicated:* generally regarded as one of the best all-round fixers; especially recommended for beginners. Gives a fine and delicate fixation in histology and cytology; used for fungi.

*Time:* small and delicate objects, 15 to 30 min.; medium objects, 1 to 2 hr.; large and dense objects, 6 hr. Do not overfix.

*Wash:* go direct from fixer into 70% iodinated alcohol and proceed as per schedule for corrosive sublimate.

*Stains:* any stains may be used.

**Orth's Fluid:** see Bichromate-Formalin, under Potassium Bichromate.

#### Osmic Acid.

*Usage:* seldom used alone, generally in one of the Flemming mixtures. Read carefully the remarks and cautions on osmic acid, p. 297. Used alone for vapor fixation and as an aqueous solution of 1 or 2%. For vapor fixation, fasten or suspend object to underside of cork and place cork in bottle tightly. If material is on a slide, invert slide over mouth of osmic bottle, preferably with the acid in the form of the dry crystals.

*Indicated:* vapor fixation—isolated cells and smears, retina of eye; solution fixation—small and thin bits of tissue for cytological work; penetration is poor.

*Time:* vapor fixation—30 sec. to a few minutes for cells and smears, 3 hr. for retina. Solution fixation, 24 hr. Well-fixed objects by either method are completely brown.

*Wash:* vapor fixation—50A, 15 min.; solution fixation, running water, 24 hr.

*Stains:* hematoxylin, carmines, safranin.

*Variant:* Lee kept his osmic acid in the form of a 2% solution in a 1% aqueous solution of chromic acid; *i.e.*, first make up a 1% aqueous solution of chromic acid. To 100 cc. of this, add 2 g. osmic acid. This stock solution may be used in making up various Flemming mixtures as well as for vapor fixation.

## Picric Acid.

### PICRIC ACID.

*Making:* used both alone and in mixtures. Alone, employed as a saturated aqueous solution; 1 g. of the crystals will saturate about 75 cc. of water.

*Indicated:* protozoa; general botany and zoology; not recommended for cytology.

*Time:* protozoa and other small objects, 1 to 2 min.; larger objects, 24 to 48 hr.

*Wash:* it is important to note that in fixation by picric acid and its mixtures, washing out is performed with alcohol, not water. Put objects in several changes of 70A until the alcohol is no longer discolored.

*Stains:* where it can be avoided, do not use aqueous staining solutions, but alcoholic instead. Water at any stage seems to undo the fixation. All stains take well after picric.

### PICRIC-ALCOHOL (Gage's Fluid).

Picric acid.....	1 part
Alcohol, 95%.....	250 parts
Water, distilled.....	250 parts

*Indicated:* good general fixer for whole mounts or tissues for sectioning.

*Time:* 1 to 3 days.

*Wash:* see Picric Acid.

*Stains:* any stain may follow, the carmines especially well.

#### PICRIC-ACETIC.

*Making:* make a 1% aqueous solution of glacial acetic acid. Saturate this with picric acid.

*Indicated:* general use; better than picric alone.

*Other Data:* same as for Picric Acid.

#### PICRIC-ACETIC-FORMALIN (Bouin's Fluid).

Picric acid, saturated aqueous solution..... 75 cc.

Formalin, commercial..... 25 cc.

Acetic acid, glacial..... 5 cc.

*Indicated:* one of the best of all general fixers; especially recommended for beginners, since almost impossible to misuse. "When in doubt, use Bouin's."

Botany, zoology, cytology.

*Time:* 6 to 24 hr., but longer stay does no harm.

*Wash:* 50A, two or more changes, 1 to 2 hr.; 70A, 1 to 2 days, several changes, or until alcohol ceases to be noticeably colored with the picric acid.

#### PICRIC-CORROSIVE (Rabl's Fluid).

Picric acid, saturated aqueous solution..... 1 part

Corrosive sublimate, saturated aqueous solution..... 1 part

Water, distilled..... 2 parts

*Indicated:* embryos.

*Time:* 12 hr.

*Wash:* 35A, several changes, 2 hr., then proceed with dehydration.

*Stains:* any stains suitable for embryos.

#### PICRIC-SULPHURIC (Kleinenberg's Mixture).

Picric acid, saturated aqueous solution..... 49 cc.

Sulphuric acid, c. p., concentrated..... 1 cc.

Water, distilled..... 100 cc.

*Indicated:* embryos; one of the best for chick embryos.

*Time:* early embryos to 48 hr. stage, 2 to 4 hr.; older embryos, 4 to 6 hr.

*Wash:* 70A, several changes, until color ceases to be extracted.

*Stains:* any desired stain may follow.

#### PLATINIC-ACETIC-OSMIC (Hermann's Fluid).

Platinic chloride, 1% aqueous solution.....	60 cc.
Osmic acid, 2% aqueous solution.....	8 cc.
Acetic acid, glacial.....	4 cc.

*Usage:* will seldom be attempted since it is the most costly of all reagents. However, is very fine in cytological work. Follow directions as for Flemming mixtures, and read instructions concerning osmic acid, p. 297.

#### Potassium Bichromate (Potassium Dichromate; Bichromate of Potassium).

##### BICHROMATE OF POTASSIUM.

*Usage:* seldom used alone as a fixer, but is one of the finest hardeners known; employed thus for the nervous system very extensively. Lee gives following data: place tissues first in a 2% aqueous solution of the bichromate, gradually increasing this strength up to 5%. A sheep's eye requires 3 wk.; spinal cord, 3 to 6 wk.; a good-sized brain, 3 to 6 mo. Then wash thoroughly in running water, after which dehydrate slowly in alcohol in the dark, beginning with a weak alcohol and passing gradually to 80A, in which the material may be stored until needed for sectioning. Change the alcohol as often as it is turned yellow. Carmine and hematoxylin stains follow well, but if carmine is to be used it should be applied as an *in toto* stain before the material is put into alcohol.

#### BICHROMATE-ACETIC (Tellyesnick's Fluid).

Bichromate of potassium.....	3 g.
Acetic acid, glacial.....	5 cc.
Water, distilled.....	100 cc.

*Making:* dissolve the bichromate in the water as a stock; add the acetic just before using.

*Indicated:* general use and embryology.

*Time:* 24 to 48 hr.

*Wash:* running water, 6 to 12 hr., and pass first to 15A in dehydrating.

*Stains:* many may follow, hematoxylin perhaps best.

#### BICHROMATE-ACETIC-FORMALIN (Smith's Fluid).

Bichromate of potassium.....	0.5 g.
Formalin, commercial.....	10.0 cc.
Acetic acid, glacial.....	2.5 cc.
Water, distilled.....	87.5 cc.

*Usage:* same as preceding.

*Indicated:* frog eggs and tadpoles.

#### BICHROMATE-CORROSIVE (Zenker's Fluid).

Bichromate of potassium.....	2.5 g.
Corrosive sublimate.....	5.0 g.
Sodium sulphate.....	1.0 g.
Water, distilled.....	100.0 cc.
Acetic acid, glacial.....	5.0 cc.

*Making:* see full description of manufacture and use, p. 161.

*Indicated:* one of the best general fixers, especially in histology and embryology.

*Time:* 12 to 36 hr., depending on size and permeability.

Tissues should not be overfixed. Guyer gives the following schedule for chick embryos: 2 to 4 hr. for 2-day chick; 8 to 10 hr. for objects or embryos of 6 to 8 mm.; 24 hr. for embryos or objects of 12 to 14 mm.

*Wash:* running water, 12 to 24 hr. For after treatment see Corrosive Sublimate.

*Stains:* practically all stains will follow Zenker's.

**BICHROMATE-CORROSIVE-FORMALIN.** See Formalin-Corrosive-Bichromate, known also as Zenker Formalin Mixtures; Helly's Fluid; Maximow's Fluid.

**BICHRIMATE-FORMALIN (Orth's Fluid).****Stock Solution**

Bichromate of potassium.....	12 g.
Sodium sulphate.....	1 g.
Water, distilled.....	900 cc.

*Making:* to 9 parts of the stock solution, add just before using, 1 part full strength commercial formalin.

*Indicated:* excellent general purpose fixer; often used in place of Zenker's since it contains no corrosive sublimate and hence the iodine aftertreatment is omitted; widely employed with nervous tissues.

*Time:* 12 to 24 hr., according to size of material.

*Wash:* running water, 6 hr.

*Stains:* any desired stain may follow.

**Rabl's Fluid :** see Picric-Corrosive.

**Schaudinn's Fluid :** see Corrosive-Acetic-Alcohol.

**Smith's Fluid :** see Bichromate-Acetic-Formalin.

**Tellyesnicky's Fluid :** see Bichromate-Acetic.

**Worcester's Fluid :** see Formalin-Corrosive.

**Zenker's Fluid :** see Bichromate-Corrosive.

**DISSOCIATORS, MACERATORS, DECALCIFIERS****Gage's Formalin Dissociator.**

Normal saline solution.....	500 parts
Formalin, commercial.....	1 part

For epithelia and nerve cells; 24 hr.

**Ranvier's One-third Alcohol.**

Alcohol, 90%.....	1 part
Water, distilled.....	2 parts

Epithelia will macerate in 24 hr.

**Müller's Fluid.****Stock Solution**

Bichromate of potassium.....	2-2½ g.
Sodium sulphate.....	1 g.
Water, distilled.....	100 cc.

This stock was formerly much used as a fixer, especially with nervous tissues. As a macerator for epithelia, 1 part of stock is mixed with 9 of normal salt solution and allowed to act 24 to 48 hr., until the cells dissociate easily. Since Müller's time, authorities agree that the sodium sulphate in this formula is useless and may as well be omitted.

### MacCallum's Macerating Fluid.

Nitric acid, concentrated.....	1 part
Glycerin.....	2 parts
Water, distilled.....	2 parts

This makes a 20% nitric acid, generally considered as the best strength for dissociating muscle fibers. Allow to act for an average time of 48 hr.

### Nitric Decalcifier.

Nitric acid, concentrated.....	10 parts
Alcohol, 70%.....	90 parts

For description of usage in decalcifying bone, see p. 296.

## STAINS

Arranged alphabetically. Cross-referenced to authors. Stains may be employed repeatedly, until they give evidence of wearing out.

**Aceto-Carmine** (Schneider).—Add carmine to boiling acetic acid of 45% until no more will dissolve;  $\frac{1}{2}$  g. or less will saturate 100 cc. of this acid. Cool and filter. For method of use, see p. 301. Widely advocated in studying nuclei and chromosomes of fresh material. See also Iron Aceto-Carmine.

**Acid Fuchsin**: see Fuchsin, Acid.

**Altmann**: see Fuchsin, Acid; and Picric Acid.

### Alum Cochineal.

Potassium alum.....	10 g.
Cochineal, powdered.....	10 g.
Water, distilled.....	150 cc.



*Making:* boil 1 hr., cool, filter. Becker and Roudabush recommend boiling the filter paper and its residue 30 min. in 75 cc. distilled water, then, after cooling and filtering, the second filtrate is added to the first and the combination boiled 30 min., cooled and filtered. This final filtrate is now made up to 150 cc. (the original volume) by adding distilled water, the completed staining solution is poured into a stock bottle, and a small crystal of thymol or a few drops of carbolic acid added to prevent mold.

*Indicated:* probably the most widely used of all whole mount stains; embryos, worms; 24 to 36 hr., according to size. Sections, 6 hr. and up.

*Aftertreatment:* wash, distilled water, 10 to 20 min., to extract the alum, but do not wash to excess. Dehydrate: whole mounts about 1 hr. in each alcohol, sections 1 min. in each. Differentiate in acid alcohol as necessary.

*Counterstains:* orange G. Lvons blue, light green, picric acid.

**Anilin Stains** (General Remarks).—The coal-tar or anilin dyes include a great many brilliant stains of all colors, used for section staining. Most of them fade in the course of time. They should be made up as fresh solutions in small amounts, as many of them do not keep for more than a few months. Those classed as basic are nuclear dyes; those marked acid are counterstains for cytoplasm. Solvents for these dyes vary widely according to preferences of different technicians: water, alcohol of any strength, anilin water, or even the clearer, for example, dissolving orange G in clove oil. Guyer recommends adding alcohol to anilin water (see p. 344) to make a 20% alcoholic solution as the best solvent for anilin dyes to be used for nuclei, following fixation of material in Hermann's or Flemming's fluids; whereas he prefers 70 to 95A as the solvent for cytoplasmic stains.

**Anilin Blue.**

*Making:* 1% solution in 90A.

*Indicated:* an acid dye used as a cytoplasmic counter-stain, generally following phloxine or safranin. Favored by botanists but seldom employed by zoologists except in such mixtures as the following entry. Cellulose walls are stained a brilliant blue; also excellent for cilia, achromatic figures, and algae. For example of usage, see p. 171.

**Anilin Blue-Orange G-Acid Fuchsin (Mallory's Triple Connective-tissue Stain).**

## Solution I

Fuchsin, acid.....	0.2 g.
Water, distilled.....	100 cc.

## Solution II

Anilin blue, water soluble.....	0.5 g.
Orange G.....	2 g.
Phosphomolybdic acid, 1% aqueous solution.....	100 cc.

*Indicated:* a beautiful combination of many contrasts for general vertebrate histology, especially for organs containing mixed tissues and for developing bone.

*Procedure:* Use an organ fixed in Zenker's or Bouin's. Stain in solution I, 5 to 15 min. Transfer directly, without washing, to solution II, 20 min., then into two changes of 90A, 30 sec. each. A third jar of 90A or 95A is now used to differentiate, as it extracts the blue. When the blue is of a satisfactory intensity, as controlled under the microscope, pass paraffin sections rapidly through 100A to xylene and mount in balsam. Transfer celloidin sections from the 90A or 95A directly into beechwood creosote for clearing, then mount in balsam.

*Results:* connective and reticular tissues, collagen fibers, mucus, amyloid, blue; nuclei and cytoplasm of cells, muscle, axis cylinders, neuroglia fibers, red; elastic fibers, pale pink or yellow; erythrocytes and myelin

sheaths, yellow. Developing bone: cartilage, light blue; bone, dark blue.

**Anilin Water.**—Solvent vehicle for making up anilin stains.

*Guyer's Method:* shake up 4 cc. of anilin oil in 90 cc. of distilled water, and filter through a wet filter. If a weakly alcoholic solution is desired, add enough 95A to make the whole mixture a 20% alcoholic solution.

*Chamberlain's Method:* make a 10% solution of anilin oil in 95A, shaking frequently and until dissolved; then add distilled water to make the whole mixture a 20% alcoholic solution.

*In either case,* 100 cc. of this solvent is generally taken with 1 g. of the dry stain to make a 1% solution of the dye.

**Auerbach:** see Fuchsin, Acid; and Methyl Green.

**Basic Fuchsin:** see Fuchsin, Basic.

**Belling:** see Iron Aceto-Carmine.

**Biebrich Scarlet.**—Highly recommended by British microscopists as an acid dye that never overstains and is not diffuse. Lee regards it as the best of all cytoplasmic stains. Use as a 1% solution.

**Bismarck Brown.**

*Making:* this dye is not easily soluble. Many technicians boil 1 g. of the dry powder in 100 cc. distilled water, then filter after a day or so, but Conn states that boiling alters the composition. Others add some acetic acid to increase the solubility, but if so made the stain is not permanent. Still others recommend adding carbolic acid in varying amounts. Perhaps the best general method is to make a saturated aqueous solution, then add one-third by volume of 90A.

*Indicated:* intra-vitam for ciliates, in which case the stain must of course be an aqueous solution, and is handled as detailed for neutral red, p. 54. For sections this is a

basic, nuclear dye that works rapidly but will not over-stain, and is permanent. Wash with 95 or 100A.

**Borax Carmine** (Grenacher).

Borax.....	4 g.
Carmine.....	1 g.
Water, distilled.....	100 cc.

*Making:* dissolve the borax; then add the carmine and heat gently until fully dissolved. When cool, add 100 cc. 70A, let stand 24 hr., and filter.

*Indicated:* for staining in toto; embryos, worms, and the like, for whole mounts, or organs for sectioning.

*Treatment:* objects taken from water or 50A are stained for several hours to several days, depending on bulk and permeability, then passed directly to acid alcohol destain and left there until color is no longer seen coming away, and the object is a bright, clear scarlet. Then complete the dehydration as usual. Sections that have been stained in bulk in this manner merely require treatment with xylene to dissolve the paraffin, then mount in balsam; if a counterstain is desired, go through xylene to 100A, and counterstain in Lyons blue or light green in 95A.

**Bordeaux Red.**—An acid, cytoplasmic stain, used as a 1% aqueous solution, 12 to 24 hr. Heidenhain found this stain, followed by his iron hematoxylin, excellent for demonstrating centrosomes in mitosis.

**Carbolfuchsin:** see Fuchsin, Basic; and Carbohc Acid.

**Carmine.**—Of the multitude of formulas employing carmine, carminic acid and carmalum, only alum cochineal and borax carmine are given. Consult Lee or other large manual for further details.

**Champy-Kull Triple Stain:** see Fuchsin, Acid; Toluidin Blue and Aurantia.

**Congo Red.**—An acid, cytoplasmic dye used as a 0.5% aqueous solution, contrasting well after hematoxylin.

Chamberlain uses a saturated solution for work in plant anatomy, staining first the cellulose walls with anilin blue or malachite green, then after a water rinse, staining in Congo red for 15 min., washing in water, transferring to 85A to differentiate, until the green or blue begins to show through the red, thence quickly through 100A to xylene and mounting in balsam.

**Conklin**: see Hematoxylin, Picro-.

**Crystal Violet**: see under Gentian Violet.

**Cyanin**.—A basic blue dye for chromosomes, becoming more and more popular in recent years in cytology. Purchase only the brand made by H. A. Metz & Co.

*Making*: dissolve 1 g. cyanin in 100 cc. 95A, then add 100 cc. distilled water.

*Procedure*: has been used for infusoria, spermatozoa, fat, lignified walls of plant tissues, and chromosomes, almost always in combination with erythrosin. Pass material from 50A into cyanin for 5–10 min., rinse quickly in 50A, stain in erythrosin 30–60 sec., again rinse rapidly in 50A, then in 95 and 100A, clearing in xylene and mounting in balsam. Both stains wash out in alcohol, hence the dehydrating must be rapid and skimpy.

*Results*: plant tissues; lignified materials blue, cellulose red. Cells; nuclei or chromosomes blue, cytoplasm pink.

**Delafield**: see Hematoxylin.

**Ehrlich**: see Hematoxylin.

**Eosin**.—The favorite counterstain after Delafield's hematoxylin in routine histologic work. Formerly existed in many varieties, some soluble in water only, others in alcohol only; now the manufacturers supply water-and-alcohol-soluble types almost exclusively, and in two principal hues, eosin bluish and eosin Y (yellowish). In buying for general work in microscopy, specify eosin Y, water-and-alcohol soluble. It is highly fluorescent and the

1 % aqueous solution makes a good red ink. Technicians differ as to preference for the solvent—water, alcohols of various strengths, or the clearer.

*Procedure:* for botanical whole mounts, as algae and fungi, use a 1 % aqueous solution and apply for 24 hr. Without washing, differentiate in a 2 % aqueous solution of acetic acid, 5 to 10 min., changing two or three times, and pass, still without washing, to 10 % glycerin, which is then allowed to concentrate. Mount in glycerin jelly or wash out the glycerin with alcohol to which a few drops of acetic acid have been added, and proceed with the venetian turpentine technique. The stain is intensified and keeps better in a slightly acidified environment.

For sections, as in vertebrate histology, use a 0.5 % solution of eosin in 95A, and apply when the slide has reached 95A during dehydration, for 30 to 60 sec., rinse in 95A and proceed with regular balsam mounting technique. Eosin is specific for erythrocytes, staining them a coppery hue, and for eosinophilic granules of certain leucocytes. It is hence a constituent of several compound blood stains, such as Wright's. Eosin is an acid, cytoplasmic dye.

### **Eosin and Methylen Blue.**

*Indicated:* a general tissue staining combination, particularly good to demonstrate the various blood corpuscles as seen in sections of organs. Especially indicated for hemolymph glands.

*Procedure:* use material fixed in corrosive sublimate or its mixtures; Zenker's best. Cut sections 5 to 6 microns thick. From water, stain sections 30 min. in 5 % aqueous solution of eosin, rinse in distilled water, then stain 15 min. in a diluted alkaline methylen blue solution. Becker and Roudabush recommend Goldhorn's polychrome methylen blue solution, obtainable from supply houses, diluted 1:10 with distilled water. See Methylen

**Blue.** Rinse in distilled water to remove excess blue and differentiate in 95A, observing under microscope to see that blue is limited to nuclei. Pass rapidly through 100A, clear in xylene, and mount in balsam.

**Erythrosin.**—A form of eosin and handled in the same manner. See also under Cyanin.

**Fast Green (FCF).**—An improved acid, cytoplasmic stain now generally replacing light green in favor, as it is much more permanent. Use as indicated for light green.

**Flemming's Triple Stain:** see Safranin-Gentian-Orange.

**Fuchsin, Acid.**—A very fine acid, cytoplasmic dye, generally made up as a 0.5% aqueous solution. Most sections are stained for 2 to 3 min., pollen grains and other entire objects, 1–2 hr. Differentiate with tap water, which is usually slightly alkaline, the stain being very sensitive to bases. Some workers wash out first in tap water, then in water with a few drops of acid to intensify the stain. Cytoplasmic and achromatic structures, mitochondria, and connective tissues are dyed red. Affords good contrast with methyl green, as in the following.

**Fuchsin, Acid; and Methyl Green** (Auerbach's Stain).

*Indicated:* for mitosis.

*Procedure:* cut thin sections, 5 microns or less, from material fixed in corrosive sublimate or its mixtures. Stain 1 hr. in a 1% aqueous solution of methyl green, wash in water until microscopic examination shows green in the chromosomes only and none in the spindle, then stain 1 min. in a 1% aqueous acid fuchsin, slightly acidulated with 2 to 3 drops of acetic acid. Go directly to 95A and observe under microscope to see that chromosomes are green, spindles red. Complete dehydration rapidly in 100A, then xylene, and balsam. If the stains do not take correctly, vary the duration of each accordingly.

**Fuchsin, Acid; and Picric Acid (Altmann).**

*Indicated:* for mitochondria.

*Procedure:* fix material for 24 hr. in mixture of equal parts 5% potassium bichromate and 2% osmic acid. Make a 5% solution of acid fuchsin in anilin water and apply this stain to a section on a slide by heating over a low flame until the reagent steams. Cool for 5 to 6 min. Prepare a saturated alcoholic solution of picric acid and dilute with 2 volumes of distilled water. Apply this one-third picric with heat, until steaming, blot, dehydrate quickly, clear in xylene, and mount in balsam.

*Results:* nuclei yellow, mitochondria bright red.

**Fuchsin, Acid; and Picric Acid (Van Giesen's Picrofuchsin).**

Fuchsin, acid, 1% aqueous solution.....	1 part
Picric acid, saturated aqueous solution.....	10 parts

*Indicated:* an excellent counterstain after hematoxylin for material fixed in any of the corrosive sublimate fixers. Used particularly for organs with an abundance of connective or nervous tissues.

*Procedure:* overstain with hematoxylin, rinse in water, and counterstain 5 min. with picrofuchsin. Add a pinch of picric acid crystals to each of the alcohols for dehydration and to the xylene. Mount in balsam.

*Results:* nuclei and epithelia, brown; white fibrous connective tissue, red; elastic tissue and muscle, yellow.

**Fuchsin, Acid; Toluidin Blue and Aurantia (Champy-Kull Triple Stain).**

*Indicated:* a fine cytological technique for protozoa, invertebrates, histology, and embryos.

*Procedure:* use small pieces of tissue fixed in Champy's Mixture and cut sections 5 microns or less. The first stain is the same as the first of Altmann's, acid fuchsin heated to steaming. Allow slide to cool for 6 min., then pour off this stain, rinse in distilled water, and counterstain in a 0.5% toluidin blue in distilled water. A



saturated aqueous solution of thionin may be used in place of the toluidin blue. Stain 1 to 2 min., wash in distilled water, then differentiate in a 0.5% solution of aurantia in 70A for 20 to 40 sec., controlling under the microscope for extraction of the fuchsin. Pass to 95A and check on extraction of the blue, thence to 100A, xylene, and balsam.

*Results:* cytoplasm, golden yellowish to green; chromatin, blue; mitochondria, and occasionally the Golgi apparatus, red. Usually the Golgi apparatus will not show except in male germ cells.

**Fuchsin, Basic.**—This is a basic, nuclear red dye, used in botany to stain lignified tissues and to demonstrate the vascular system of living plants. Of several methods for this latter purpose, that of Gourley consists of removing a plant from the soil, washing the roots, immersing them in the stain, and cutting off part of the roots beneath the stain. After 24 to 48 hr. the whole vascular system will be stained red, when the plant may be dissected under magnification, or dehydrated and cleared in xylene by small and slow changes over 10 to 12 hr. His formula: dissolve 50 mg. of basic fuchsin in 2 cc. 95A, then dilute with 100 cc. of tap water.

As a general tissue stain in histology, basic fuchsin may be applied in 1% aqueous solution for 24 hr., rinsed in 50A, and the sections counterstained 5 min. in methylen blue, giving chromatin and nucleoli red and other structures blue. More often basic fuchsin is employed in the form of specialized compound stains, as the two following.

**Fuchsin, Basic; and Carbolic Acid** (Ziehl-Neelsen's Carbol-fuchsin).

Fuchsin, basic, saturated solution in 95A.....	10 cc.
Carbolic acid, 5% aqueous solution.....	90 cc.

• *Making:* about 0.3 g. of basic fuchsin will saturate 10 cc. of 95A, according to Guyer.

*Indicated:* bacteria, many forms. Demonstrates spores and acid-fastness. Keeps well, acts vigorously and is used with both smears and sections.

*Procedure:* see p. 197.

**Fuchsin, Basic; and Resorcin (Resorcinfuchsin; Weigert's Elastic Tissue Stain).**

Fuchsin, basic, 1% aqueous solution.....	100 cc.
Resorcin, 2% aqueous solution.....	100 cc.

*Making:* heat the two solutions together in a porcelain vessel. When boiling, add 25 cc. of a 30% solution of ferric chloride and continue boiling 5 min. Cool, filter, and discard the liquid. Boil the precipitate in 200 cc. 95A until dissolved. Cool and filter again, and add enough 95A to make up the loss; up to 200 cc. Then add 4 cc. hydrochloric acid. If the prepared dry stain, resorcinfuchsin, is used, make first a 1% solution of hydrochloric acid in 95A, then saturate this solution with the dye. Crystal violet may be substituted for the fuchsin.

*Procedure:* use formalin fixed material containing elastic tissue, such as aorta, large arteries, elastic cartilage, or lung. Stain sections 15 to 45 min., wash in 95A, dehydrate in 100A, clear in xylene, and mount in balsam.

*Results:* elastic fibers, dark blue.

*Combinations:* may be preceded by alum cochineal or hematoxylin or followed by picrofuchsin to provide contrast.

**Gentian Violet.**—This is a famous basic, nuclear anilin dye, but will probably be discontinued before long under this name as it is not a pure substance, but a mixture of crystal violet and a methyl violet. According to Conn, the stain to use in bacteriology and where a bluish shade of violet is preferred in histology and cytology, as is most often the case, is crystal violet; if a reddish hue is

preferred in histology and cytology, methyl violet 2B. Gentian violet stains powerfully and keeps well.

*Indicated:* bacteriology, as in the Gram stain and capsule stain; histology and cytology, both plant and animal; also in a number of celebrated combinations.

*Making:* use the anilin-water solution for work in bacteriology or cytology; a saturated aqueous solution for ordinary histological purposes.

*Procedures:* (1) Bacteria: the anilin-water gentian violet solution may be used as a simple stain, as described on p. 186, or as the more exact Gram stain in which the violet is followed by treatment with Lugol's iodine solution and finally a counterstain with safranin or basic fuchsin, detailed on p. 186. The capsule stain is given on p. 198. (2) Chromosomes: thin sections from material fixed in a Flemming or other osmic acid fixer are brought down to water and stained 5 to 15 min. in gentian, the time depending mainly on the age of the stain; freshly made gentian staining more rapidly than an older solution. Rinse in water and mordant sections 1 to 3 min. in Lugol's solution until they turn black, then into 100A until they are gray. Pass to clove oil to differentiate. The alcohol alone may be used for this purpose but extracts the stain so rapidly that most technicians prefer to go from 100A into clove oil, since this extracts the dye more slowly and thus gives one better control; also the action is more differential. Chamberlain states that the stain may be completely removed from chromosomes while still bright in achromatic structures so that by following up with safranin one can get red chromosomes on a violet spindle. Go through two or three clearings in xylene and mount in balsam. (3) Gatenby recommends a combination with picric acid: mordant 10 to 20 min. first in the iodine solution, rinse in water, stain 15 min. in gentian, rinse in water, iodine solution again 1 min., 95A rinse, then flood with saturated solution of picric

acid in 100A, dip in 100A, differentiate in clove oil, and clear thoroughly in several changes of xylene, not less than 15 min. (4) Bouin fixed material: stain in gentian 15 min., mordant in iodine solution 2 min., dip in 100A, then in a 1% solution of chromic acid in water for 15 sec., 10 sec. in 100A, and differentiate in clove oil as in method 3. (5) Chamberlain uses gentian for cilia, starch grains, and cellulose walls. In combination with safranin he obtains red lignified walls and violet cellulose walls.

**Gold Chloride.**—Used for metallic impregnation, chiefly for nerve fibers, as detailed on p. 307. Many additional techniques will be found in the larger manuals.

**Gram's Iodine Solution:** see Iodine Solution.

**Gram Stain.**—Bacteria; see p. 186.

**Grenacher:** see Borax Carmine.

**Heidenhain:** see Hematoxylin, Iron.

**Hemalum** (Mayer).—A coined name from hematein and alum. See general remarks under Hematoxylin.

**Making:** (1) from hematoxylin crystals. Dissolve 1 g. of these crystals in 1 l. of distilled water. Add 0.2 g. sodium iodate and 50 g. alum (aluminum ammonium sulphate); dissolve and filter. Add a crystal of thymol as a preservative. (2) From hematoxylin stock solution. Take 10 cc. of this stock and 1 l. of distilled water, proceeding exactly as in using the crystals, method 1.

**Indicated:** used like Delafield's hematoxylin but has the advantage of being ready for use as soon as made, and is also recommended for toto staining. Good for algae and fungi to be mounted in glycerin.

**Procedure:** in concentrated form hemalum stains very rapidly, 1 to 10 min. for sections, 1 to 12 hr. for whole mounts, according to size. Dilutions are easier to control, however. Dilute 2:1 with distilled water and

stain sections 1 hr., whole amounts 24 hr.; or dilute 10:1 and stain sections from several hours to overnight. For whole mounts, wash in running water about the same length of time as used for staining; 10 to 15 min. for sections. Sections are best not overstained, but if this happens, follow the differentiation procedure as for Delafield's hematoxylin. Clear in xylene and mount in balsam. Can be followed by any acid counterstain of suitable color, as acid fuchsin, eosin, erythrosin, or fast green.

**Hematoxylin** (General Remarks).—This is a natural dye extracted from Brazilian logwood and has long been a favorite in the arts as well as by far the most important of all stains in microscopy. As worked out by Mayer, the true dye is not hematoxylin itself but an oxidation product termed "hematein," which combines with alum to form a blue stain. A mordant is necessary to make the dye "take"; it prepares the tissue to combine with the dye, which forms what is termed a "lake"—an insoluble aluminum-hematein salt that will not wash out. In some of the many formulas, as Delafield's, the mordant and dye are combined in a single solution; in others, as iron hematoxylin, the mordant precedes as a separate reagent.

Theoretically, hematein alone should be used instead of hematoxylin; in actual practice it does not work so well and nearly all technicians prefer the lengthier process of ripening hematoxylin solutions by exposure to air (oxidation) to produce hematein. As Lee states, there is no way of ascertaining, except by repeated trial, when such a solution is ready to give a good stain; during the weeks or months consumed in the oxidation process there will be mixtures of unripe, ripe, and overripe constituents, the first and last of which are valueless for staining purposes. Nevertheless this seemingly illogical procedure continues to give the best results.

The various formulas are mostly made up from a stock solution, made by dissolving 1 part of hematoxylin crystals in 10 parts of absolute alcohol, *e.g.*, 10 g. crystals in 100 cc. alcohol. Where possible, this solution should be made up well in advance of use—months or even a year—during which time it slowly oxidizes and becomes wine red. Solutions made from ripe stock will be ready for use immediately. Anyone doing a considerable amount of staining should see that his supply of hematoxylin solution is kept about one year ahead of his staining schedule, making up a fresh, dated stock every 6 mo. to provide future replacements of that used meanwhile.

Various methods of speeding up the ripening process have been used; see under Delafield's Hematoxylin. The hematoxylin stains at their best following fixation in any chromic mixtures, but will follow almost any fixing solution. The chief mordants used with this dye are aluminum, chromium, iron, and copper.

Hance adds a small amount of bicarbonate of soda to hematoxylin solutions, finding that this improves both the staining action and keeping properties.

#### HEMATOXYLIN, CONKLIN'S PICO-

*Making:* Delafield's hematoxylin is diluted with 4 parts distilled water to one of the hematoxylin; for every cc. of the mixture, one drop of Kleinenberg's Picric-Sulphuric fixing solution is added.

*Indicated:* particularly good with embryos.

*Procedure:* for whole mounts, stain 1 to 3 hr.; for embryos that are to be sectioned, stain them 12 hr. (Guyer).

#### HEMATOXYLIN, DELAFIELD'S.

*Making:* saturate ~~100~~ cc. distilled water with alum, using approximately 20 g. aluminum ammonium sulphate. Dissolve 1 g. hematoxylin crystals in 10 cc. 100A and add this drop by drop to the alum

solution. Place in a narrow-necked bottle and leave uncorked in the sunlight for several weeks to ripen. Six weeks is a good time; two months not too long. Or use 10 cc. of ripened hematoxylin stock solution. In either case, when the mixture is ripe, filter and add 25 cc. glycerin and 25 cc. pure methyl alcohol. The next day filter again and pour into a stock bottle which is henceforth kept tightly stoppered.

*Quick Ripening:* this and other hematoxylin solutions may be ripened at once with more or less success by several methods, the best, if one has access to the proper equipment, being that of Neild, who used ultraviolet light, as that from a Cooper-Hewitt or other powerful mercury-vapor lamp. The alum and hematoxylin solution is exposed in a shallow dish at a distance of 2 to 3 feet for 1 hr.; the complete stain, after the other ingredients have been added, exposed for 2 hr. more. Mayer and Unna, independently, found out that neutralized hydrogen peroxide or other strong oxidizing agent will almost instantaneously ripen hematoxylin, and Piazza gives 7% as the right amount of peroxide to add for Delafield's, 12% for Ehrlich's. Harris makes up his Delafield's as follows: dissolve 1 g. hematoxylin crystals in 10 cc. absolute alcohol (or take 10 cc. of the stock solution) and add this to a warm solution of 20 g. alum in 200 cc. distilled water. Heat until boiling and add 0.5 g. mercuric oxide. Boil 1 min., then plunge the flask into cold water, and continue to cool rapidly under the cold-water faucet. The mercuric oxide effects the ripening and the stain is ready for immediate use. Just before using, add 4% glacial acetic acid.

*Indicated:* much the preferred formula for routine and general use, in both botany and zoology. Any contrasting acid counterstain may follow, eosin being most commonly favored.

*Procedure:* see detailed schedule, p. 276. Dilute one-half or more with distilled water before using.

### HEMATOXYLIN, EHRLICH'S ACID-

Hematoxylin.....	2 g.
Acetic acid, glacial.....	10 cc.
Potassium alum.....	10 g.
Alcohol, absolute.....	100 cc.
Glycerin.....	100 cc.
Water, distilled.....	100 cc.

*Making:* dissolve the hematoxylin in the alcohol, then add the acid, then the glycerin. Separately dissolve the alum in the water by heating and, while still warm, pour this slowly into the hematoxylin mixture while stirring. Expose in an uncorked bottle to light and air for 3 to 6 wk., until of a deep red color, then cork tightly. This solution needs no filtering and keeps for years.

*Indicated:* gives a reddish rather than a bluish stain; even so contrasts well with eosin. Does not over-stain, hence is good for staining in bulk, while the procedure with sections is simpler. Preferred by many to Delafield's.

*Procedure:* stain for 5 to 30 min., depending on the object, inspecting occasionally to note progress. Slides may go into this stain from 50 or 35A, and are washed afterward with the same or even 70A. Differentiation, as with Delafield's, is not necessary. Counterstain with eosin, erythrosin, fast green, or orange G.

### HEMATOXYLIN, HEIDENHAIN'S IRON-

#### Solution I

Ferric alum.....	2 g.
Water, distilled.....	100 cc.

#### Solution II

Hematoxylin, 0.5% aqueous solution.

*Making:* the ferric alum indicated for this work is ferric ammonium sulphate; do not confuse with the ferrous salt. The crystals should be of a clear violet



color; if green, they are of the wrong salt; if yellowed or opaque, they are too old and have deteriorated. Solution I is best when fresh, and deteriorates after some 2 months. Recommended strengths vary from 1.5 to 4%; 2 to 2.5% are best for general purposes. Solution II can be made by dissolving 0.5 g. hematoxylin crystals in 10 cc. of absolute or 95A, then adding the water; or by taking some of the stock 10% solution and diluting it 1:19 with distilled water. In either case the hematoxylin must be ripe before using.

*Procedure:* see the example given on p. 167. Sections are transferred from water into solution I, in which they are mordanted for an average duration of 6 hr.; overnight is not too long in a 2% ferric alum. They are then washed thoroughly in distilled water, 10 min. to 1 hr., changing several times, as any excess alum carried over into the stain is harmful. Stain in solution II, 1 to 12 hr., 6 hr. again being an average; the slides are purposely overstained. Rinse well in tap water, then place in a second—fresh—jar of the mordant, solution I, to differentiate. Run one slide through first as a trial horse and watch the destaining at intervals under the microscope, being sure to keep the material wet with water during such inspections. When the stain is of correct intensity, remove the slide and wash in running water for 1 to 2 hr., as it is essential to extract all of the iron alum. Dehydrate, clear, and mount as usual. If a counterstain is wanted, use eosin, erythrosin, fast green, or orange G.

*Modifications:* see under Hematoxylin (general remarks) the method of Hance, adding some bicarbonate of soda to the stain. Many workers today prefer to differentiate in a saturated aqueous solution of picric acid instead of in a second jar of solution I, then washing out 2 hr. in running water. This method destains the cytoplasm and leaves chromosomes sharply defined.

*Indicated:* the most important of all staining techniques in cytology; should be thoroughly mastered by all microscopists. See various uses, p. 168.

### HEMATOXYLIN, MALLORY'S PHOSPHOTUNGSTIC ACID-

Hematoxylin, crystals.....	0.1 g.
Water, distilled.....	80.0 cc.
Phosphotungstic acid, 10% solution (Merck).....	20.0 cc.
Peroxide of hydrogen (U.S.P.).....	0.2 cc.

*Making:* dissolve the hematoxylin in the water with the aid of heat, then add the acid, and then the peroxide to ripen the mixture; or omit the peroxide and allow the solution to ripen with the lapse of time.

*Indicated:* a very fine cytological technique to demonstrate centrosomes and spindles in mitotic figures. In histology it is used for connective tissues, muscles, and many kinds of fine fibers.

*Procedure:* Becker and Roudabush list the following steps: material is fixed in Zenker's and processed through sectioning as usual, and the slides carried to water. Potassium permanganate, 0.25% solution, 4 min.; wash, distilled water; oxalic acid, 5%, 5 to 10 min.; distilled water, five changes, 1 min. each; phosphotungstic hematoxylin, 12 to 24 hr.; wash quickly in distilled water; 95A, 1 min.; 100A, 1 min. or less; xylene, 3 min.; mount in balsam.

*Results:* a polychrome stain; nuclei, blue, intercellular substances, pink.

### Iodine Solution (Lugol's Solution; Gram's Solution).

#### Lugol's Formula

Iodine.....	4 g.
Iodide of potassium.....	6 g.
Water.....	100 cc.

#### Gram's Formula

Iodine.....	1 g.
Iodide of potassium.....	2 g.
Water.....	300 cc.

#### Kent's Formula

Make a saturated solution of potassium iodide in distilled water, then saturate this with iodine, filter, and dilute to a brown sherry color.

*Uses:* Kent's formula is a good fixing agent for protozoa. Lee uses either Kent's or Lugol's for small marine animals, making up the solutions with sea water, and has found Lugol's excellent for spermatozoa. Gram's formula is generally favored as a mordant in bacteriology, though some prefer the stronger Lugol's.

### **Iron Aceto-Carmine (Belling).**

*Making:* dissolve some ferric hydrate in 45% acetic acid and add a very little, 1, 2, or 3 drops, to Schneider's aceto-carmine, making the latter bluish red but without causing any precipitation. This makes chromosomes stain more deeply. Then add to this treated stain an equal amount of untreated stain, stir to mix, and employ as detailed on p. 301.

*Indicated:* nuclei and chromosomes of fresh cells. See also Aceto-Carmine (Schneider). A blue-green filter aids the studying of aceto-carmine preparations under the microscope.

### **Iron-Alum Mordant for Bacteria.**

Ferric chloride, 5% aqueous solution.....	25 cc.
Alum (aluminum ammonium sulphate) saturated aqueous solution.....	75 cc.
Fuchsin, basic, saturated aqueous solution.....	10 cc.

*Making:* mix the chloride and alum and shake well, then add the fuchsin, filter, and allow to stand 1 to 2 weeks before using. For procedure, see p. 198.

**Janus Green.**—An important *intra-vitam* dye, the technique for which is described under Neutral Red. Mitochondria in living cells will stain with the Janus Green B obtainable from L. A. Metz & Co., New York (Cowdry), diluted 1:500,000 with normal saline. More often technicians employ strengths of 1:30,000 or 40,000 for mitochondria; still others use 1:15,000 or 20,000, depending on the type of dye, the organism, and the cells selected for treatment.

**Light Green** (Lichtgrün S. F.).—A very beautiful green counterstain that may follow any basic dye of suitable contrast in hue, but particularly noted in combination with safranin (see p. 299). It is an acid, cytoplasmic dye that does not keep well; see Fast Green. It is soluble in either water or alcohol, but most technicians prefer a 0.5% solution in 95A, staining for but a few seconds as it works very rapidly.

**Loeffler**: see Methylen Blue, Alkaline.

**Lugol's Solution**: see Iodine Solution.

**Lyons Blue** (Bleu de Lyon).—An anilin blue, this dye provides a beautiful acid, cytoplasmic, contrast stain after carmine or safranin. Recommended especially for embryos, nervous tissue, and cartilage. Usually made up as a 0.5% solution in 95A; average staining time for sections, 15 sec.

**Malachite Green**.—A basic dye primarily of use in botany to color cellulose walls. Stain sections in a 3% aqueous solution of malachite green 6 hr. or longer, wash in water, counterstain 15 min. in 1% aqueous Congo red, wash again in water, and differentiate in 80A until the green appears through the red, then dehydrate quickly in 100A, clear in xylene, and mount in balsam (Gregoire's method).

**Mallory**: see Anilin Blue, Orange G, Acid Fuchsin (triple connective-tissue stain); or Hematoxylin, Phosphotungstic Acid-.

**Mayer**: see Hemalum; Paracarmine.

**Methylen Blue**.—The literature on this dye is very extensive; for fuller treatment see one of the large manuals, as Lee's *Vade-Mecum*. Methylen blue is a basic stain, not to be confused with methyl blue, an acid dye.

*Types*: Alkaline methylen blue (Loeffler) is widely used in bacteriology. Make first a saturated solution of the stain in 95A; 1 g. of the dry powder will saturate about

100 cc. alcohol. Next dissolve 1 part caustic potash in 10,000 parts water. Add 30 cc. of the staining solution to 100 cc. of the potash solution.

In botany, methylen blue may be substituted for malachite green in the work described under the latter stain.

The medicinal grade is a pure stain and the one to use for most purposes, especially for *intra vitam* and staining of nervous tissues.

Polychromatic methylen blue contains methylenazur as an oxidation product; although this substance would be regarded as an impurity for many types of work, it is desirable in others, especially in cytology, as it affords a differential coloring of certain cell granules.

*Methods:* routine; botany or zoology. As a nuclear dye, use in the way given for safranin. For general botanical work, Barratt stains sections in polychrome methylen blue, then mordants for a few minutes in a 10% aqueous solution of ammonium molybdate before dehydrating.

Unna stains histologic sections containing visceral muscle in polychrome methylen blue, rinses in water, immerses for 10 min. in 1% aqueous solution of potassium ferricyanide, decolorizes in acid alcohol, then continues in the regular manner.

Dogiel impregnates epithelia, lymph spaces, and other constituents of a piece of thin membrane by immersing the fresh tissue for a few minutes in 4% methylen blue in normal saline solution. Fix for 30 to 45 min. in a saturated aqueous solution of ammonium picrate, wash in a fresh amount of the same, and examine in glycerin. For a permanent mount, use equal parts of pure glycerin and the ammonium picrate solution. Let the tissue stand in this for 24 to 48 hr.; then mount in a fresh portion of the mixture and seal on a turntable.

*Intra-vitam* staining of small, transparent, aquatic organisms is done by adding enough of the stain to the water containing the organisms to give it a light blue shade.

Nervous tissue may be treated by injection or immersion. In the first case, if a small animal is used, it is chloroformed and the body cavity or a main artery injected with a 1% solution of the dye in normal saline. With larger animals, inject the artery leading to the desired organ. Repeat the injection after 15 min., then 30 min. later dissect the organ and remove small bits of the tissue, including its nerve connections, and tease on a slide wet with normal saline. It is important to allow free exposure of the tissue to air as oxidation must take place. Examine frequently under the microscope; when the desired elements are well stained, fix them quickly or the stain will fade rapidly. Fix by immersion for 24 hr. in a saturated aqueous solution of ammonium picrate and proceed by Dogiel's method (opposite page) for a permanent mount.

In the immersion method small bits of fresh tissue on a slide are treated with a 0.1% solution of the dye in normal saline. Put a few drops of this dilution on the material at 3 min. intervals, but do not cover with the fluid as exposure to air for oxidation is required. Continue as per the injection method from this point on.

**Methyl Green.**—An important basic, nuclear, anilin dye.

*Indicated:* (1) for mitosis; see Auerbach's stain; (2) for chromatin of fresh material, either whole small organisms, as protozoa, or isolated tissues.

*Procedure:* make up a strong aqueous solution, one in which the final color is dark green, and add 1% acetic acid. All solutions used with this stain should be acidulated. Methyl green kills cells instantly, without swelling or distortion. It is specific for chromatin, which

it colors immediately and never overstains. If permanent slides are wanted, the water for washing and the alcohols should be acidulated, and the alcohols and balsam should be colored with the stain as otherwise fading is rapid. Even then such mounts are not very satisfactory.

Fixed tissues may be stained, but see that the fixer is pure corrosive sublimate.

**Methyl Violet:** see Gentian Violet.

**Neutral Red.**—A basic dye, chiefly of use for *intra-vitam* staining, as described on p. 54. For fixed material a 1% aqueous solution is employed.

**Orange G.**—One of the preferred acid, cytoplasmic counter-stains, used in many combinations. Widely employed following iron hematoxylin; does not overstain; is slow in action; does not keep well, hence make up in small amounts and use fresh. The favored formula is a 1% aqueous solution; some workers prefer alcohol or clove oil as the solvents. For the last named, Chamberlain recommends dissolving 0.1 g. orange in 100 cc. 100A, then adding 100 cc. clove oil. Allow to evaporate until the whole is down to 100 cc., thus getting rid of the alcohol.

**Orcein.**—A stain derived from a lichen and used mainly to demonstrate elastic fibers in connective tissue. Unna stained sections for 10 min. in polychrome methylen blue, then washed in water, blotted off excess, treated 15 min. in a neutral 1% solution of orcein in 100A, rinsed in 100A, cleared in bergamot oil, and mounted in balsam.

**Osmic Acid.**—One form of preparation to demonstrate certain fats, as in ordinary adipose tissue, and the myelin sheaths of voluntary nerve fibers is to blacken these substances by fixation of the tissue in 1% osmic acid (see under Killing and Fixing Agents). A small nerve, freshly removed from an animal, may be fixed in 1% osmic 24 hr., washed in running water 24 hr., then removed to a

slide, and teased to display fibers. Dehydrate, clear in clove oil, and mount in balsam.

**Paracarmine** (Mayer).

Carminic acid.....	1.0 g.
Aluminum chloride.....	0.5 g.
Calcium chloride.....	4.0 g.
Alcohol, 70%.....	100 cc.

*Making:* dissolve the solids in the alcohol, allow to settle, then filter.

*Indicated:* for protozoa, sections, and large whole objects. Should not be used with materials having an alkaline reaction or containing lime, such as sponge spicules.

*Procedure:* for large protozoa, dilute one-half with 70A and acidify with a few drops of glacial acetic acid. Stain overnight and wash with acid alcohol, 70%, which will also differentiate. Sections and whole objects are stained overnight, then washed out with 70A; if overstained, add 0.5% aluminum chloride or 2.5% glacial acetic acid to this washing alcohol.

**Phloxine.**—One of the eosins, an acid dye. Highly recommended by Chamberlain for whole mounts of algae, counterstained with anilin blue; see p. 171. For algae a 1% solution in 90A is used; for sections, dilute this solution one-half with water. Stain 24 hr. and rinse in 95A.

**Picrofuchsin** (Van Giesen's): see Fuchsin, Acid and Picric Acid.

**Picro-Indigo-Carmine.**—One of the best of the very numerous combinations of a dye with picric acid. Made up in a number of proportions: for embryos, a mixture of equal parts of saturated aqueous solutions of picric acid and indigo-carmine is preferred; for protozoa, use 3 parts of the stain to 1 of picric, both saturated in 70A; other workers employ 2 parts dye to 1 of picric, in either water or alcohol.

*Indicated:* an excellent counterstain following basic fuchsin



(see p. 319), carmine, safranin, magenta, or iron hematoxylin.

### **Picro-Nigrosin.**

Nigrosin, saturated aqueous solution.....	1 part
Picric acid, saturated aqueous solution.....	9 parts

*Indicated:* films or sections of protozoa and invertebrates.

*Procedure:* use a sublimate fixer and avoid Bouin's fluid.

After carmine staining and a wash in distilled water, counterstain with picro-nigrosin 5-10 min., wash 1 min. in tap water, then dehydrate rapidly in 70 and 90A and continue as usual to a balsam mount, or go directly into euparal. Successful results are very handsome.

**Resorcinfuchsin:** see Fuchsin, Basic and Resorcin.

**Safranin.**—Doubtless the most important and widely employed of all anilin stains; a basic, nuclear, and chromatin dye of many uses. Of the many varieties, the safranin O of Grübler is the best and most reliable.

*Making:* a very large number of formulas may be found in advanced manuals. Saturated or 1% aqueous solutions are generally designated in the various double and triple staining combinations, such as the safranin-gentian-orange described on p. 299. Babe's fluid is popular and consists of a mixture of equal parts of saturated aqueous and saturated alcoholic solutions of the dye. Many workers prefer an anilin water combination, Guyer's formula being:

Safranin.....	1 g.
Anilin water.....	90 cc.
Alcohol, 95%.....	10 cc.

Chamberlain often uses a 1% safranin in 50A. To 100 cc. of a 1% solution in 100A, Flemming added after 4 days, 20 cc. distilled water.

*Indicated:* general botany—roots, stems, and leaves—the favorite combination being safranin and light green, as detailed on p. 299; histology—isolated nerve cells,

sense organs, spermatozoa; cytology—chromosomes, mitosis.

*Procedure:* safranin is a powerful, brilliant, and durable stain and the solutions keep well. However, it is rapidly washed out in the alcohols during dehydration and hence overstaining is generally practiced. If differentiation is required, use untreated alcohol for resting nuclei, acid alcohol for dividing nuclei. Such acid alcohol must be very weak, as 1 part of acid to 1,000 of alcohol. If the safranin-light green combination is used, keep in mind that the counterstain will extract the safranin rapidly; accordingly pass sections into the green while the safranin is still intense. The general staining time is 24 hr. Wash according to the vehicle for the stain; if an aqueous solution, wash in water; if an alcoholic solution, wash in alcohol of the strength used in making the stain.

*Counterstains:* light green, fast green, gentian violet, orange G, anilin blue, Delafield's hematoxylin.

**Safranin-Gentian-Orange** (Flemming's Triple Stain).—See p. 299.

**Scharlach R.**—A specific stain for fat cells and a micro-chemical test for fats.

*Making:* mix equal parts of acetone and 70A, then saturate this mixture with the dry stain.

*Procedure:* fix material for sectioning in formalin; cover glass films in formalin vapor. Use the freezing method of sectioning, either before or after fixation in formalin, as alcohol dissolves fat and the other machine microtome methods are hence inadmissible. Dip a section for a moment in 50A, then stain for 10 to 15 hr., rinse in 70A, wash in water, and mount in glycerin or glycerin jelly. Fat cells are bright red. If it is desired to counterstain the surrounding tissues, methylen blue is good. Treat cover glass films similarly but stain a shorter time, as 5 min. See also p. 295.

**Schneider** : see Aceto-Carmine.

**Silver Nitrate.**—The best-known and most widely used of the metallic impregnation methods, for demonstrating intercellular substances, cell walls, and nerve cells and fibers. The silver salt is reduced by exposure to sunlight, in an action like that of exposing a photographic plate, and a brown or black deposit is formed in the designated materials.

*Methods*: various workers use aqueous solutions ranging from 0.1 to 1 %, over periods of 1 to 5 min., or until the tissue is sufficiently blackened. See the following descriptions for details: cell walls of epithelia, p. 293; Golgi method for nerve cells, p. 306; Cajal's routine for nerve fibers, p. 307.

**Sudan III.**—A specific stain for fat requiring the same general methods and precautions as given under Scharlach R. Fix tissues in formalin, section by the freezing method, stain 5 to 10 min., rinse in 70A, wash well in water, and mount in glycerin or glycerin jelly. The stain is made up as a saturated solution in 82A. Prolonged exposure to the stain or other alcoholic reagent will dissolve out the fat.

### **Tannic Acid Mordant (Bacteria).**

Tannic acid, 25% aqueous solution.....	20 cc.
Ferrous sulphate, saturated aqueous solution.....	10 cc.
Fuchsin, basic, saturated solution in 95A.....	2 cc.

*Method*: described on p. 198.

**Thionin.**—A basic, nuclear dye very similar in nature and use to methylen blue. Thionin or toluidin blue are now used by many workers in preference to methylen blue as differentiation during dehydration is much slower and therefore more easily controlled.

*Making*: use a saturated aqueous solution for chromosomes; add two drops of this to 5 cc. water to make a stain for mucin.

*Indicated*: as for methylen blue; *intra-vitam*, impregnation

of cell walls and tissue spaces, chromosomes, mucin, nervous tissue, muscle.

**Procedures:** for staining differentially the mucous secretions of gland cells, as in a section of the colon, fix the tissue 5 hr. in 5% sublimate and stain the sections 5 to 15 min. in the diluted dye. Result: mucin, red; all other substances, blue. For chromosomes, use the concentrated solution and stain 5 to 10 min. King added 1% carbolic acid to the saturated aqueous solution of thionin to prevent fading and render the stain permanent. For *intra vitam*, use a concentration of 1:1,000 normal saline.

**Toluidin Blue.**—Entirely similar to the foregoing in applications and use. Harris gives the following formula for making the stain:

Toluidin blue, 0.1% solution in normal saline.....	2 parts
Ammonium chloride, 0.25% aqueous solution.....	1 part
Egg albumen.....	1 part

**Van Giesen's Picrofuchsin:** see Fuchsin, Acid and Picric Acid.

**Wright's Stain.**—A modification of Leishman's Romanowsky stain for blood preferred by most American technicians.

**Making:** three procedures are available:

1. Manufacture the complete stain yourself; for details, see Mallory or Guyer. The process is complicated and difficult; for most persons method 2 or 3 is advised.
2. Purchase the prepared stain in the form of a dry powder. Make a 5% solution of this in pure methyl alcohol. Keep the stock bottle well stoppered to prevent evaporation; should any precipitation occur, filter and add 1 cc. of methyl alcohol. Allow to ripen 1 month before using.
3. Purchase the prepared staining solution and use undiluted.

**Procedure:** see p. 174.

**Indicated:** for blood films and malarial parasites.

**Ziehl-Neelsen's Carbolfuchsin:** see Fuchsin, Basic and Carbolic Acid.

### DESTAINS AND BLEACHERS

**Acid destains** may be made up in water or in alcohols of varying strengths and with either hydrochloric or acetic acids, the former generally preferred. Furthermore, the concentration of acid may vary between 0.1 and 1%, giving a wide latitude in choice of the differentiating agent. Probably the most widely used destain is 0.5% hydrochloric acid in 70A, but there is hardly a set practice. Naturally, the stronger the destain the more rapid its action; duration of the destaining bath generally runs from 30 sec. to 2 min., but must be determined by a trial slide when using unfamiliar techniques. It is essential to remove all acid from the tissues following destaining as otherwise most stains will fade rather rapidly. A wash in several changes of water is usually recommended; a more careful routine employs alkalized water or alcohol (according to the make-up of the destain) to ensure neutralizing any remaining trace of acid. Such a procedure is essential in the Delafield's hematoxylin technique.

**Alcohols** used in dehydrating frequently act as destains, especially with the basic anilin stains. For example, it is advisable to have material overstained with safranin before beginning dehydration, as a certain amount of the dye will be extracted even when the alcohol series and time durations are shortened as much as possible. This fact must be taken into account in deciding whether or not to use a destain.

**Alkaline** water or alcohol, although not a destain, is discussed here in conjunction with the use of acid destains. Very often tap water is sufficiently alkaline and will blue hematoxylin slides properly, or differentiate other aqueous staining solutions. Your local tap water may be tested with litmus paper to discover whether or not it is alkaline.

If neutral or insufficiently alkaline, use one of the following:

1. Make a 0.1% solution of sodium bicarbonate in distilled water. Add 3 to 5 drops of this weak solution to the water or alcohol rinse. Larger amounts of the salt are apt to make tissues muddy.
2. Scott's tap water substitute:

Potassium bicarbonate.....	2 g.
Magnesium sulphate.....	20 g.
Water, distilled.....	1,000 cc.

Add a crystal of thymol to prevent the growth of molds.

**Chlorine** is one of the preferred bleaching agents and the fumes are readily prepared in the small quantities needed without personal risk. Mayer placed a few crystals of potassium chlorate, the size of a match head or less for the total quantity, in a test tube or small stender, and added 2 or 3 drops of hydrochloric acid. As soon as the greenish chlorine gas appears, pour in 5 to 10 cc. 50A, which dissolves the fumes. Transfer material into this from 70A and leave until bleached, which may take from only 15 min. to as much as 2 days, depending on the object. Wash well in several changes of 30A for 2 to 4 hr. before passing to water for toto staining. Sections placed in this chlorinated alcohol will bleach in 10 min. or less.

**Clearers** sometimes destain, as is true with anilin and clove oil. If such a clearer is used, allowance for some extraction of anilin dyes should be made.

**Gold Chloride** was found to be a good bleaching agent by Beams for material fixed in osmic acid. He used a 0.5% for 12 to 24 hr.

**Hydrogen Peroxide** in 3% aqueous or alcoholic solutions is a favorite bleacher. A few minutes suffices for sections, hours for toto mounts; long immersions will produce maceration.

**Mordants** often destain and differentiate, as is the case in the regular technique for iron hematoxylin.

**Potassium Chlorate** : see Chlorine.

**Potassium Permanganate and Oxalic Acid** are preferred by some for decolorizing osmic fixed material. Just before staining, sections are passed into 0.1 % potassium permanganate, 1 min., then into 0.5 % oxalic acid, 1 or 2 min., and washed well before going into the stain.

**Stains** may extract the color of previous stains by a method termed substitution. Thus, as noted by Lee, gentian violet will be discharged from tissues by adding aqueous eosin. Light green takes out safranin, though how much of this action is due to the stain and how much to the alcohol in which it is dissolved has not been determined.

**Sulphurous Acid** was used by Gilson for decolorizing bichromate-fixed material. Add 2 to 4 drops of hydrochloric acid to 10 cc. of 2 % bisulphate of sodium and put tissues into this for 6 to 12 hr.

#### NORMAL SALINE SOLUTIONS

Expert tissue culture work is carried on by using various substances in which living cells will absorb nutriment and thrive. There are many such media, including the amniotic liquor from large mammalian embryos as obtained at slaughterhouses, aqueous humor from fresh beef eyes, various blood sera, and other natural fluids, as well as artificial media of complex composition, with a carefully adjusted pH, such as tyrode. If you are interested in any of these, consult larger manuals or special books on the subject.

In this section, listing is confined to the more ordinary solutions that approximate the saline body fluids, easily made, and in which fresh cells and tissues may be examined or rinsed, and worm parasites maintained for observation in the living state.

**General Note.**—Formulas are given on a basis of 1,000 cc. of the solvent (water) so as to avoid small fractions of the salts. Either smaller or larger quantities can be made,

according to need and facilities for weighing out the solids.

### **Normal Saline; Physiological Saline or Salt Solution.—**

Preferences of individual workers vary from a 0.6% to 0.9% solution, but the following is perhaps best for general work:

Sodium chloride.....	7.5 g.
Water, distilled.....	1,000.0 cc.

**Ringer's Solutions** have long been favorites. They are superior to normal saline since they approximate more closely the blood serum of animals.

Sea Water Ringer's	
Sea water.....	1 part
Distilled water.....	3 parts
Ringer's for Cold-blooded Vertebrates	
Sodium chloride.....	8.0 g.
Potassium chloride.....	0.2 g.
Calcium chloride.....	0.2 g.
Sodium bicarbonate.....	0.2 g.
Water, distilled.....	1,000.0 cc.

Add the calcium chloride last to avoid precipitation of insoluble calcium carbonate. If tissues or animals are to be kept alive for several hours, add to the above 1 g. of dextrose (glucose).

**Ringer's for Warm-blooded Vertebrates, Locke's Solution, and Ringer-Locke.**

All are quite similar:

Sodium chloride.....	9.00 g.
Potassium chloride.....	0.42 g.
Calcium chloride.....	0.24 g.
Sodium bicarbonate.....	0.20 g.
Water, distilled.....	1,000.00 cc.

Add the calcium chloride last, according to preceding formula. Dextrin, 2.5 g., may be added as a nutrient. Neither of the artificial Ringer's keeps well and they should be made up fresh.

### **CLEARERS, AFFIXATIVES, AND MOUNTANTS**

**Carbolxylene** (carbolxylol) is a substitute for absolute alcohol in completing dehydration, plus the clearer xylene.



Pure carbolic acid or phenol is crystalline and must be melted carefully over a low flame, avoiding the fumes or any spilled liquid as these are very poisonous. Mix 1 part of the acid with 3 parts xylene. This mixture remains fluid at room temperatures.

**Glycerin Jelly** is a mounting medium, solid at room temperatures.

Gelatin, French, best quality.....	10 g.
Glycerin.....	70 cc.
Water, distilled.....	60 cc.

Mix these ingredients in a beaker standing in a dish of hot water, or use a water bath or double boiler. Dissolve the gelatin in the water, then mix in the glycerin. Add a few drops of carbolic acid to prevent growth of molds. Avoid overheating, which may convert the gelatin into metagelatin, a form that remains fluid at ordinary temperatures. To use, melt a small bit of the jelly on a slide.

**Mayer's Albumen Affixative.**—Beat up the white of one egg with a fork or egg beater and add an equal quantity of glycerin after the albumen has settled to a fluid. To prevent mold, add 1 g. of salicylate of soda or a few crystals of thymol. Filter the whole through a number of thicknesses of cheesecloth and, since this will take hours or days, keep the filter covered to exclude dust. For method of use see p. 248.

**Szomdathy's Glycerin-Gelatin Affixative.**—Place 1 g. best French gelatin in 100 cc. distilled water and heat to 30° C., stirring until dissolved. Add 1 g. carbolic acid and filter while still hot. Then add 15 cc. glycerin. To use, float paraffin ribbon sections on coated slides which have been flooded with distilled water containing 4% formalin. When sections have stretched out fully, tilt slide to drain off excess fluid, or remove with lens paper. This is a firmer affixative than the preceding and the mixture will not "fry" while warming, as is often the case with albumen.

**INJECTION MASSES**

**Ranvier's Carmine-Gelatin Mass.**—Soak 5 g. best French gelatin 24 hr. in distilled water, then melt it in the water it has absorbed by heating in a beaker standing in a dish of hot water or over a water bath. Prepare carmine by rubbing  $2\frac{1}{2}$  g. in 5 cc. distilled water, then adding ammonia drop by drop until a transparent red solution is obtained. Add this carmine solution slowly, with continual stirring, to the fluid gelatin until smoothly dissolved.

The mixture must now be neutralized by slowly and cautiously adding, drop by drop, and with continual stirring, a 25 % acetic acid. As neutrality is approached, a slight opaqueness is evident, and the odor of ammonia is gradually replaced by one faintly acid. This is an important but difficult point to detect; too much acid will precipitate the carmine and spoil the preparation; too little will give a mass that diffuses through the walls of blood vessels and stains all the adjacent tissues. Litmus paper will be of assistance here.

Inspect a drop of the finished product under the microscope to make certain that the carmine has not precipitated. If satisfactory, strain through a piece of clean new flannel.

**Berlin Blue Mass.**—Prepare a saturated aqueous solution of Berlin blue and add to a gelatin mass until of a bright blue color and such that the whole will form a jelly when cold.

**Starch Masses.**—For purposes of anatomical dissections, dissolve 40 g. cornstarch in 75 cc. 5 % formalin and add 10 cc. glycerin, stirring until smooth. Then mix in 10 g. of the pigment, which may be vermilion, chrome yellow, chrome green, or still others. For histological purposes, a more suitable mass is made by adding very finely powdered cornstarch or lampblack to a gelatin mass.



## APPENDIX A

### SOURCES OF SUPPLIES

A limited listing is given of some of the chief firms from which the microscopist may obtain the materials he needs.

- A. S. Aloe Co., 1819 Olive St., St. Louis. Clinical laboratory supplies.
- Atlas Surgical Supply Co., 175 Second Ave., New York City. New and reconditioned microscopes, accessories, surgical instruments.
- Badger Biological Supply Co., 2494 S. Fifth St., Milwaukee. Biological supplies.
- Bausch & Lomb Optical Co., Rochester, N. Y. All forms of optical equipment.
- R. & J. Beck, Ltd., 69, Mortimer St., London, W. 1, England. All forms of optical equipment.
- Biological Supply Co., 1176 Mount Hope Ave., Rochester, N. Y. Biological supplies, prepared slides.
- Burroughs Wellcome & Co., Inc., 9-11 E. 41st St., New York City. Research materials, parasites, reagents; mfg. *Soloid* tablet stains.
- California Biological Service, 1612 W. Glenoaks Blvd., Glendale, Calif. Living insects, especially parasites.
- Cambosco Scientific Co., 37 Antwerp St., Brighton Station, Boston. Biological supplies.
- Carbide and Carbon Chemicals Corp., 30 E. 42d St., New York City. Dioxan, cellosolve; in quantity.
- Cardevaant Laboratories, Edwardsburg, Mich. Microscope slides, especially histology and embryology.
- Carolina Biological Supply Co., Elon College, N. C. Biological supplies, prepared slides.
- Central Scientific Co. (Cenco), 1700 Irving Park Blvd., Chicago. General apparatus, supplies, chemicals.
- Chicago Apparatus Co., 1735-1743 N. Ashland Ave., Chicago. Apparatus, supplies, chemicals, biological materials.
- Clay-Adams Co., Inc., 117-119 E. 24th St., New York City. Apparatus, supplies.
- Kenneth R. Coe, 2024 Sunnyside Ave., Chicago. Aquiculture and biological supplies; slides.
- Denoyer-Geppert Co., 5235-5257 Ravenswood Ave., Chicago. Biological materials and equipment; microscope slides.

Difco Laboratories, Inc., 920 Henry St., Detroit. Bacteriological media and materials.

E. I. duPont de Nemours & Co., Inc., Wilmington, Del. Manufacturing chemists; solvents, plastics, isobutyl methacrylate.

Eimer & Amend, 205-223 Third Ave., New York City. Apparatus, glassware, chemicals.

Empire Laboratory Supply Co., Inc., 507-559 W. 132d St., New York City. Apparatus, glassware, chemicals.

Evans' Biological Supply Co., Box 1656, St. Petersburg, Fla. Biological material, chiefly subtropical.

Henry George Fiedler, 31-33 E. 10th St., New York City. Scientific periodicals and books, new and used.

Fisher Scientific Co., 709 Forbes St., Pittsburgh. Apparatus, glassware, chemicals.

Flatters & Garnett, Ltd., 309, Oxford Road, Manchester, 13, England. Optical instruments, apparatus, accessories, biological supplies, prepared slides, euparal, synthetic neutral mountant.

Alden H. Forbes Laboratories (Forbio), 1710 Renton Ave., West View, Pittsburgh. Biological materials, prepared slides.

Albert E. Galigher, Inc., 1228-1230 Solano Ave., Berkeley, Calif. Prepared microscope slides.

General Biological Supply House, Inc., (Turtox), 761-763 E. 60th Pl., Chicago. Apparatus, supplies and chemicals; biological materials, microscope slides; reagents for the newer techniques in small quantities; plastic micro cover slips.

C. M. Gilbert, Jr., University Station, Box 1453, Charlottesville, Va. Zoological material.

Gits Molding Corp., 4600 W. Huron St., Chicago. Razor-nife.

C. P. Goerz American Optical Co., 317 E. 34th St., New York City. Optical and photographic equipment; pocket microscopes.

Gradwohl Laboratories, 3514 Lucas Ave., St. Louis. Stains.

Graf-Apsco Co., 109 W. Hubbard St., Chicago. Microscopes, new and rebuilt; accessories, supplies.

Gruebler stains. Those prepared by J. & A. Schmid, Leipzig, should be ordered from Akatos, Inc., 55 Van Dam St., New York City; those made by K. Hollborn & Sons, Leipzig, from Pfaltz & Bauer, Inc., Empire State Bldg., New York City.

H. Randolph Halsey, 3804 Greystone Ave., New York City. Protozoan cultures and slides.

Prof. Robert T. Hance, 1 Broadmoor Ave., 16, Pittsburgh. Histo-covers (plastic film substitutes for cover glasses).

Hartman-Leddon Co. (Harleco), 6010 Haverford Ave., Philadelphia. Chemicals, reagents, parstains.

- Romeyn B. Hough Co., Lowville, N. Y. Tree and wood materials of all sorts, including microscope slide sections.
- Hynson, Wescott & Dunning, Inc., Charles & Chase Sts., Baltimore. Chemicals, staining solutions.
- Illinois Biological Supply Co. (Bico), 4834-4836 Foster Ave., Chicago. Biological supplies, slides.
- Kansas City Biological Supply Co., 3605 State Line, Kansas City, Mo. Pig embryos; frog developmental series.
- E. Leitz, Inc., 730 Fifth Ave., New York City. All forms of optical equipment; diaphanol.
- Marine Biological Laboratory, Supply Dept., Woods Hole, Mass. Biological material, slides; chiefly marine.
- M. S. Markle, Earlham College, Earlham, Ind. Botanical material and microscope slides.
- Martin Laboratories, Ovid, Mich. Microtechnical chemicals, stains, biological supplies.
- Microchemical Service, Douglaston, N. Y. Apparatus and supplies for microchemical work.
- The Neville Co., Neville Island, Pittsburgh. Clarite.
- New York Scientific Supply Co. (Nyssco), 111-113 E. 22d St., New York City. Apparatus, biological materials, supplies, slides.
- Pacific Biological Laboratories, Pacific Grove, Calif. Biological materials, slides.
- Parasitology Supply Co., 3700 40th Ave., Minneapolis. Parasitic specimens, slides.
- Pfaltz & Bauer, Inc., Empire State Bldg., New York City. Apparatus, chemicals; American distributors for C. Reichert, Vienna, optical instruments, and for Dr. Gruebler's stains manufactured by K. Hollborn and Sons, Leipzig.
- Powers and Powers, Box 1092, Lincoln, Nebr. Prepared microscope slides.
- Puget Sound Biological Supply Co., 1605 E. 47th St., Seattle. Biological materials, especially Pacific marine forms.
- Radio Corporation of America (RCA), Camden, N. J. Electron microscopes.
- Raygram Corporation, 425 Fourth Ave., New York City. Weigh-spoon.
- C. Reichert, Vienna, Austria. Microscopes and accessories. See Pfaltz & Bauer.
- A. I. Root Co., Medina, Ohio. Apiary and entomological supplies and equipment; bees, living and preserved.
- Harry Ross, 70 West Broadway, New York City. Microscopes and other scientific instruments and equipment, new and used.
- E. H. Sargent & Co., 155-165 E. Superior St., Chicago. Apparatus, chemicals, stains.

- Southern Biological Supply Co., Inc., 517 Decatur St., New Orleans  
Biological materials, slides.
- South-Western Biological Supply Co. (Texana), Box 4084, Dallas.  
Biological materials.
- Spencer Lens Co., Buffalo, N. Y. All forms of optical equipment.
- Standard Scientific Supply Corp. (Stanscien), 34-38 W. Fourth St.,  
New York City. Apparatus, supplies, biological materials.
- Arthur H. Thomas Co., West Washington Square, Philadelphia. General  
laboratory apparatus, supplies, stains, chemicals, euparal.
- Triarch Botanical Products, Ripon, Wis. Preserved plants; botanical  
microscope slides.
- Villa Alba Laboratory, Delphi, Ind. Living animals, microscope slides,  
*Drosophila* cultures.
- August Waeldin, Inc., 10 Maiden Lane, New York City. Optical instruments  
and supplies.
- Ward's Natural Science Establishment, Inc., 302 Goodman St. N.,  
Rochester, N. Y. Biological, entomological and geological supplies  
and materials; microscope slides.
- W. Watson & Sons, Ltd., 313, High Holborn, London, W. C. 1, England.  
All forms of optical equipment; microscope slides.
- W. M. Welch Scientific Co., 1517 Sedgwick St., Chicago. Scientific  
instruments, apparatus, supplies, chemicals.
- Will Corporation, Rochester, N. Y. General laboratory apparatus, supplies,  
stains, chemicals; diaphane; *Bioid* reagents and stains.
- Williams, Brown & Earle, Inc., 918 Chestnut St., Philadelphia. Scientific  
instruments, apparatus, biological supplies.
- Wistar Institute, 36th St. and Woodland Ave., Philadelphia. Experimental  
rats and mice; slide trays.
- Wollensak Optical Co., Rochester, N. Y. Miniature microscopes.
- Woodbury Label Co., Woodstock, Vt. Printed gummed labels.
- J. E. Young, 137 Pierce St., West Lafayette, Ind. *Drosophila* slides.
- Carl Zeiss, Inc., 485 Fifth Ave., New York City. All forms of optical  
equipment.
- Zoological Research Supply, Englewood, Fla. Biological materials,  
especially parasitology.
- Zoological Station, Naples, Italy. Preserved Mediterranean animals;  
embryos.

## APPENDIX B

### LITERATURE OF MICROTECHNIQUE

In prosecuting any line of work with the microscope, general or special, background preparation is of the highest importance. For this reason the references given are classified by subject and are not limited to microscopy proper. Since a complete listing of even current publications in these many fields would require a separate volume, our entries have been pruned to include only representative works. Many well-known and excellent books have been omitted, but it is felt that those here given will meet the requirements of most readers.

In the case of periodicals, continuance of publication of those of foreign origin is uncertain under present world conditions.

#### PERIODICALS

**Scientific Journals.**—These are mostly the official publications of societies. The annual membership fee includes subscription to the journal.

American Microscopical Society, *Transactions*. Published by the Secretary of the Society and hence the place of issue changes. Quarterly, 1878 to present. Chief American professional journal; advanced, technical.

American Society of Amateur Microscopists, *Bulletin*. Chestertown, Md., 1939 to present; quarterly. Aims to appeal to all classes from beginners to advanced workers.

*The Lens*, publication of the State Microscopical Society of Illinois, new series, issued irregularly at present. J. E. Nielsen, President, 5517 Drexel Ave., Chicago.

*The Microscope and Entomological Monthly*. Arthur Barron, Ltd., 61-62, Chancery Lane, London, W. C. 2, England. American agent, Harry Ross, 70 West Broadway, New York; 1937 to present. Intermediate.

Optical Society of America, *Journal*. Published by the American Institute of Physics, 175 Fifth Ave., New York; 1917 to present; monthly. Professional, advanced.

*Quarterly Journal of Microscopical Science*. Oxford University Press, 114 Fifth Ave., New York; 1853 to present. British professional and advanced periodical.



Quekett Microscopical Club, *Journal*. Published by the Club, 11, Chandos St., Cavendish Square, London, W. 1, England. Began 1868; new series 1882 to present; three issues yearly. Intermediate.

*Review of Scientific Instruments*, published by the American Institute of Physics, 175 Fifth Ave., New York; 1930 to present; monthly. Advanced.

Royal Microscopical Society, *Journal*. B. M. A. House, Travistock Square, London, W. C. 1, England; 1878 to present; bimonthly. Advanced.

*Science*, official publication of the American Association for the Advancement of Science, Science Press, Lancaster, Pa. New series 1895 to present; weekly. Frequently has advanced technique articles.

*Stain Technology*. Published by the Commission on Standardization of Biological Stains, Geneva, N. Y., 1926 to present; quarterly. Advanced.

### **Popular Magazines.**

*Nature Magazine*, 1214 Sixteenth St. N.W., Washington, D. C. Official organ of American Nature Association. Natural history and conservation; includes a department on amateur microscopy. Ten issues per year.

*Popular Science Monthly*, 381 Fourth Ave., New York. Includes an article each month on amateur microscopy.

**Trade Journals or House Organs.**—These range from brief notes to more or less elaborate and scientific magazines and are issued monthly or quarterly. They are sent without charge to teachers, libraries, museums, and microscope clubs, and to individuals who prove to be regular customers. The average individual cannot purchase a subscription. Articles deal with many phases of science and usually include features on microscopy, microtechnique, and optics as well as collecting, classifying, and preparing biological materials, along with commercial announcements of prepared slides. For addresses of publishers, see Appendix A.

*Biology Briefs*, Denoyer-Geppert Co.

*Carolina Tips*, Carolina Biological Supply Co.

*Cenco News Chats*, Central Scientific Co.

*Educational Focus*, Bausch & Lomb Optical Co.

*Englewood Trade Wind*, Zoological Research Supply.

*The Laboratory*, Fisher Scientific Co.

*Notebook*, Chicago Apparatus Co.

*Triarch Topics*, Triarch Botanical Products.

*Turtox News*, General Biological Supply House.

*Ward's Natural Science Bulletin*, Ward's Natural Science Establishment.

*Watson's Microscope Record*, W. Watson & Sons, Ltd.

## BOOKS

### Anatomy.

*Comparative, vertebrate.*

1. WALTER, H. E., *Biology of the Vertebrates*, Macmillan, New York, 1939. A celebrated comparative anatomy written so that anyone can understand it.

*Frog.*

2. SHUMWAY, W., *The Frog*, Macmillan, New York, 1928. Directions for dissecting this favorite laboratory animal.

*Cat.*

3. REIGHARD, J., and H. S. JENNINGS, rev. by R. Elliott, *Anatomy of the Cat*. Holt, New York, 1935. Standard full treatment for this much-used type.

*Pig.*

4. SINCLAIR, J. G., *Anatomy of the Fetal Pig*, Collegiate Press, Ames, Iowa, 1936. Complete, illustrated directions for dissection.

*Rat.*

5. HUNT, H. R., *A Laboratory Manual of the Anatomy of the Rat*, Macmillan, New York, 1924. Instructions for dissection.

*Man.*

6. WILLIAMS, J. F., *A Textbook of Anatomy and Physiology*, Saunders, Philadelphia, 1939. The best treatment of this very comprehensive subject within the limits of one smallish volume.

### Bacteriology.

7. BERGEY, D. H., *Manual of Determinative Bacteriology*, Williams & Wilkins, Baltimore, 1939. Standard advanced work for classification.
8. CONN, H. W., rev. by H. J. Conn, *Bacteria, Yeasts and Molds in the Home*, Ginn, New York, 1932. Good intermediate work.
9. EYRE, J. W. H., *Bacteriological Technique*, Williams & Wilkins, Baltimore, 1930. Advanced, comprehensive reference on methods.
10. STITT, E. R., *Practical Bacteriology, Haematology and Animal Parasitology*, Blakiston, Philadelphia, 1938. A famous work on identification, laboratory procedures, and diagnoses; reference manual for the advanced laboratory technician.
11. SWINGLE, D. B., *General Bacteriology*, Van Nostrand, New York, 1940. Recommended as among best of modern texts; handsome, interesting volume.

**Biology.***General.*

12. MAJOR, J. W., *General Biology*, Macmillan, New York, 1941. A leading college text, very readable, excellent for background.

*Aquatic.*

13. NEEDHAM, J. G., and P. R. NEEDHAM, *A Guide to the Study of Fresh-Water Biology*, Comstock, Ithaca, N. Y., 1935. Small booklet, profusely illustrated, excellent for identification of microscopic aquatic organisms.
14. WARD, H. B., and G. C. WHIPPLE, *Freshwater Biology*, Wiley, New York, 1918. A classic; the bible of aquatic biology.

**Botany.***General.*

15. GRAY, A., rev. by B. L. Robinson and M. L. Fernald, *New Manual of Botany*, American Book, New York, 1908. For many years the best general work for identification.
16. TRANSEAU, E. N., H. C. SAMPSON, and L. H. TIFFANY, *Textbook of Botany*, Harper, New York, 1940. Most beautiful and one of the best and most readable of recent botanies; recommended for background.

*Algae.*

17. MACBRIDE, T. H., and G. W. MARTIN, *The Myxomycetes*, Macmillan, New York, 1934. Standard work on the slime molds.
18. SMITH, G. M., *Fresh-water Algae of the United States*, McGraw-Hill, New York, 1933. Outstanding manual needed by all workers in this field.
19. TIFFANY, L. H., *Algae, the Grass of Many Waters*, Thomas, Springfield, Ill., 1938. For all interested in algae.

See also 13, 14.

*Diatoms.*

20. BOYER, C. S., "Synopsis of the North American Diatomaceae," *Proceedings of the National Academy of Sciences*, Philadelphia, vols. 78-79 and supplements, 1927. Comprehensive treatment; may be consulted in the larger libraries.

See also 13, 14, 18, 19.

*Fungi.*

21. ARTHUR, J. C., *Manual of the Rusts in the United States and Canada*, Purdue Research Foundation, Lafayette, Ind., 1934. Taxonomic.
22. BESSEY, E. A., *Textbook of Mycology*, Blakiston, Philadelphia, 1939. Comprehensive advanced treatment of the fungi.
23. HENRICI, A. T., *Molds, Yeasts and Actinomycetes*, Wiley, New York, 1930. Systematic study of fungi of great importance to the microscopist.

See also Bacteriology; 8.

**Pollen.**

24. WODEHOUSE, R. P., *Pollen Grains*, McGraw-Hill, New York, 1935. Complete work by the recognized authority.

**Wood.**

25. BROWN, H. P., and A. J. PANSHIN, *Commercial Timbers of the United States*, McGraw-Hill, New York, 1940. Chief text for identification of timbers with microscopes.

**Chemistry, including staining.**

26. CHAMOT, E. M., and C. W. MASON, *Handbook of Chemical Microscopy*, 2 vols., Wiley, New York, 1938-1939. Invaluable reference work, recent and comprehensive.
27. CONN, H. J., *Biological Stains*, Commission on Standardization of Biological Stains, Geneva, N. Y., 1936. Technical reference; chief source book on stains and staining.
28. CONN, H. J. and others, *The History of Staining*, Geneva, N. Y., 1933. Authoritative work on the subject; bibliography.
29. HILL, D. G., J. H. SAYLOR, W. C. VOSBURGH, and R. N. WILSON, *Elementary Chemistry*, Holt, New York, 1937. Excellent modern college text, recommended for background.

See also 32.

**Criminology—Scientific Crime Detection.**

30. HATCHER, J. S., *Textbook of Firearms Investigation, Identification and Evidence (and) Textbook of Pistols and Revolvers*, Small-Arms Technical Pub. Co., Marines, Onslow County, N. C., 1936. Widely used reference in ballistics.
31. KUHNE, F., *The Finger Print Instructor*, Scientific American Pub. Co., New York, 1935. Good guide for this study.
32. LUCAS, A., *Forensic Chemistry and Scientific Criminal Investigation*, Longmans, New York, 1935. Textbook of the modern Sherlock Holmes; outstanding work.
33. OSBORN, A. S., *Questioned Documents*, Boyd Printing Co., Albany, N. Y., 1929. Out of print, but still available from dealers; important for those interested in microscopy of handwriting, type-writing, and allied phases of criminology.

**Crystallography.**

34. BENTLEY, W. A., and W. J. HUMPHREYS, *Snow Crystals*, McGraw-Hill, New York, 1931. The standard work; over 2,000 photomicrographs.
35. DALE, A. B., *The Form and Properties of Crystals*, Macmillan, New York, 1932. Small volume with minimum essentials of crystallography.

**Cytology.**

36. SHARP, L. W., *Introduction to Cytology*, McGraw-Hill, New York, 1934. Standard advanced work on cellular structure and function. See also 104.

**Dictionary, scientific.**

37. *Chambers's Technical Dictionary*, ed. by C. F. Tweney and L. E. C. Hughes, Macmillan, New York, 1940. Excellent reference.

**Drawing, scientific.**

38. MUELLER, J. F., *A Manual of Drawing for Science Students*, Farrar, New York, 1935. Splendid brief treatment; can be used and enjoyed by anyone.

**Embryology.**

39. ADAMSTONE, F. B., and W. SHUMWAY, *A Laboratory Manual of Vertebrate Embryology*, Wiley, New York, 1939. Guide to anatomy of selected ages of frog, chick, and pig embryos.
40. HUETTNER, A. F., *Fundamentals of Comparative Embryology of Vertebrates*, Macmillan, New York, 1941. New college text, recommended for thorough preparation.

**Entomology.**

41. COMSTOCK, J. H., *An Introduction to Entomology*, Comstock, Ithaca, N. Y., 1940. Standard advanced text.
42. JAKES, H. E., *How to Know the Insects*, pub. by author, 709 N. Main St., Mt. Pleasant, Iowa, 1939. Humorous pictured key for beginners.
43. LUTZ, F. E., *Field Book of Insects*, Putnam, New York, 1935. Excellent pocket manual for identification; intermediate.
44. Ward's Natural Science Establishment, Inc., *How to Make an Insect Collection*, Ward's Natural Science Establishment, Inc., 302 Goodman St. N., Rochester, N. Y., 1940. Valuable illustrated booklet on methods.

See also 65, 69, 89, 90, 105, 106, and Parasitology, animal.

**Geology.***General.*

45. CRONEIS, C., and W. C. KRUMBEIN, *Down to Earth*, Univ. of Chicago Press, Chicago, 1936. Excellent college text, recommended for background.

*Mineralogy and Petrography.*

46. JOHANNSEN, A., *Essentials for the Microscopical Determination of Rock-forming Minerals and Rocks*, Univ. of Chicago Press, Chicago, 1928. Small volume of great utility.

47. ROGERS, A. F., and P. F. KERR, *Thin-Section Mineralogy*, McGraw-Hill, New York, 1933. Identification and optical properties of minerals in rocks; methods; advanced.
48. WINCHELL, A. N., *Elements of Optical Mineralogy*, an introduction to microscopic petrography, 3 vols., Wiley, New York. Part 1, Principles and Methods, 5th ed., 1937; Part 2, Descriptions of Minerals, 3d ed., 1933; Part 3, Determinative Tables, 2d ed., 1940. Outstanding reference work.

#### *Soils.*

49. KUBIĚNA, W. L., *Micropedology*, Collegiate Press, Ames, Iowa, 1938. Expert treatment of soil microscopy.

### **Histology.**

50. BREMER, J. L., *Textbook of Histology*, Blakiston, Philadelphia, 1936. One of the most widely used college texts.
51. COLE, E. C., *Text-book of Comparative Histology*, Blakiston, Philadelphia, 1941. Very modern, brief, well illustrated, and with the newer techniques.
52. SCHAFER, E. S., *Essentials of Histology*, Lea & Febiger, Philadelphia, 1938. One of the finest volumes in this field; complete; advanced.
53. STILES, K. A., *Handbook of Microscopic Characteristics of Tissues and Organs*, Blakiston, Philadelphia, 1940. Splendid supplement to any text; facilitates learning; enables identification.

### **History, science.**

54. CLAY, R. S., and T. H. COURT, *History of the Microscope*, Griffin, London, 1932. One of the latest treatments of the subject.
55. SEDGWICK, W. T., and H. W. TYLER, rev. by H. W. Tyler and R. P. Bigelow, *A Short History of Science*, Macmillan, New York, 1939. Best one-volume work on history of all sciences.

See also 28.

### **Industrial Microscopy.**

#### *General.*

56. HANAUSEK, T. F., transl. by A. L. Winton, *The Microscopy of Technical Products*, Wiley, New York, 1907. Though out of date, still one of the best general reference manuals in this field.

#### *Dairy.*

57. American Public Health Association, *Standard Methods for the Examination of Dairy Products*, New York, 1939. Splendid advanced reference.

#### *Foods.*

58. WOODMAN, A. G., *Food Analysis*, McGraw-Hill, New York, 1931. Detailed discussion, microscopy, photomicrographs.

*Metals.*

59. HOYT, S. L., *Metallography*, 2 vols., McGraw-Hill, New York, 1920. Comprehensive, advanced microscopic study of metals.
60. SAUVEUR, A., *The Metallography and Heat-Treatment of Iron and Steel*, McGraw-Hill, New York, 1935. Standard advanced manual.

*Paper.*

61. CALKIN, J. B., *Microscopy of Paper*, Tappi, New York, 1937. Fine guide; bibliography.

*Textiles.*

62. MATTHEWS, J. M., *Textile Fibers: Their Physical, Microscopical, and Chemical Properties*, Wiley, New York, 1924. A well-known text and reference.
63. SCHWARZ, E. R., *Textiles and the Microscope*, McGraw-Hill, New York, 1934. Complete yet concise manual covering all phases of the subject; a standard reference.

*Water.*

64. WHIPPLE, G. C., rev. by G. M. Fair and M. C. Whipple, *Microscopy of Drinking Water*, Wiley, New York, 1927. Best known volume on the subject; advanced; good bibliography.

*Wood:* See 25.**Laboratory Techniques**, other than microtechnique.

65. American Association for the Advancement of Science, Committee of Section F, J. G. Needham, Chairman, *Culture Methods for Invertebrate Animals*, Comstock, Ithaca, N. Y., 1937. Advanced and highly useful collection of culturing methods.
66. CAMERON, G., *Essentials of Tissue Culture Technique*, Farrar, New York, 1935. Excellent manual for those unfamiliar with this procedure; bibliography.
67. GRADWOHL, R. B. H., *Clinical Laboratory Methods and Diagnosis*, Mosby, St. Louis, 1938. Basic reference manual for hospital and laboratory technicians.
68. MALLORY, F. B., *Pathological Technique*, a practical manual for workers in pathological histology, including directions for the performance of autopsies and for microphotography, Saunders, Philadelphia, 1938. A famous standard work; earlier editions were by Mallory and Wright.
69. *Turtlox Service Leaflets*, General Biological Supply House, Chicago. Various numbers include many excellent aids for the microscopist in collecting, identifying, culturing, and processing. The firm will send a list of these leaflets on request.

See also 9, 10, 44, 95.

**Microscope.**

70. ALLEN, R. M., *The Microscope*, Van Nostrand, New York, 1940. Excellent recent work for the general reader; bibliography.
71. BECK, C., *The Microscope; Theory and Practice*, Van Nostrand, New York, 1938. Advanced optical theory clearly explained; one of the best of the briefer works.
72. BELLING, J., *The Use of the Microscope*, McGraw-Hill, New York, 1930. Advanced exercises in critical microscopy.
73. CARPENTER, W. B., rev. by W. H. Dallinger, *The Microscope and Its Revelations*, Blakiston, Philadelphia, 1901. Out of print. One of the most famous older works; large, many illustrations.
74. CORRINGTON, J. D., *Adventures with the Microscope*, Bausch & Lomb Optical Co., Rochester, N. Y., 1934. Introduction to materials and methods of the several sciences using the microscope.
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See also 6.

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See also 13, 14, 89, 90.

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See also 1.

## APPENDIX C

### REFERENCE TABLES

#### MEASUREMENTS OF MASS

##### Apothecaries' Weights

###### *Table of Units*

20 grains (gr.) or minims (℥) = 1 scruple
3 scruples (℥) = 1 dram (also spelled drachm) = 60 grains
8 drams (dr.) = 1 ounce = 24℥ = 480 grains
12 ounces (oz.) = 1 pound (lb.) = 96 dr. = 288℥ = 5,760 grains

*Use:* for weighing drugs; United States.

*Standard:* the ounce, which is the troy ounce; see under Troy Weights.

*Equivalents:*

1 scruple = 1.296 grams
1 dram = 3.888 grams

For correspondence of grains, ounces, and pounds with units of other systems, see under Troy Weights.

##### Avoirdupois Weights

###### *Table of Units*

16 drams (dr.) = 1 ounce
16 ounces (oz.) = 1 pound (lb.) = 256 drams

*Use:* for weighing all commodities except precious stones, precious metals, and drugs; English-speaking countries.

*Standard:* the pound. In the United States the standard example is preserved at the National Bureau of Standards, Washington. It is defined as 0.453593 of the standard kilogram.

*Equivalents:*

1 dram = 27.344 grains
1 ounce = 437.5 grains
1 pound = 7,000 grains
1 dram = 1.772 grams
1 ounce = 28.35 grams
1 pound = 453.593 grams
1 pound = 0.454 kilogram
1 ounce = 0.912 ounce troy
1 pound = 1.215 pounds troy

**Metric Weights***Table of Units*

1 milligram (mg.)	= 1 cubic millimeter (cu. mm.).
1,000 milligrams	= 1 gram = 1 cubic centimeter (cc.).
1,000 grams (g.)	= 1 kilogram (kg.).

*Use:* optional in the United States for avoirdupois or troy; universally employed in all sciences.

*Standard:* the gram, defined as the thousandth part of the standard kilogram.

*Equivalents:*

1 milligram	= 0.015 grain
1 gram	= 15.432 grains
1 gram	= 0.257 dram apothecaries'
1 gram	= 0.564 dram avoirdupois
1 gram	= 0.035 ounce avoirdupois
1 gram	= 0.643 pennyweight troy
1 gram	= 0.032 ounce troy
1 kilogram	= 35.274 ounces avoirdupois
1 kilogram	= 2.205 pounds avoirdupois
1 kilogram	= 32.151 ounces troy
1 kilogram	= 2.679 pounds troy

**Troy Weights***Table of Units*

24 grains (gr.)	= 1 pennyweight
20 pennyweight (dwt.)	= 1 ounce = 480 grains
12 ounces (oz.)	= 1 pound (lb.) = 240 dwt. = 5,760 grains

The ounce is now more usually divided decimally.

*Use:* for weighing gold, silver, and all precious stones except diamonds and pearls; England, the United States, etc.

*Standard:* the pound or ounce, originally based on grains.

*Equivalents:*

1 grain	= 64.799 milligrams
1 grain	= 0.065 gram
1 pennyweight	= 1.555 grams
1 pennyweight	= 0.878 dram avoirdupois
1 ounce	= 31.104 grams
1 ounce	= 1.097 ounces avoirdupois
1 pound	= 373.242 grams
1 pound	= 0.373 kilogram
1 pound	= 0.823 pound avoirdupois

**MEASUREMENTS OF CAPACITY****Apothecaries' Liquid Measure***Table of Units*

## SYSTEM 1

60 minims (℥)	= 1 dram
8 drams (℥ i)	= 1 ounce
16 ounces (℥ i)	= 1 pint
8 pints (O i)	= 1 gallon (C i)

## SYSTEM 2

60 minims (℥)	= 1 fluid dram
8 fluid drams (f ℥)	= 1 fluid ounce
16 fluid ounces (f ℥)	= 1 pint
8 pints (O)	= 1 gallon (Cong.)

Dram is also spelled "drachm."

*Use:* pharmaceutical fluid measures in the United States.

*Standard:* the gallon. In the United States this is defined as containing 231 cu. in.

<i>Equivalents:</i>	1 minim	=	0.062 cubic centimeter
	1 fluid dram	=	3.697 cubic centimeters
	1 fluid ounce	=	29.572 cubic centimeters
	1 pint	=	473.152 cubic centimeters
	1 pint	=	28.875 cubic inches
	1 pint	=	0.473 liter
	1 gallon	=	231 cubic inches
	1 gallon	=	3.785 liters
	1 gallon	=	8.339 pounds avoirdupois
	1 gallon	=	10.134 pounds troy

The last two items above convert gallons to mass and are figured for distilled water at 4° C. with the barometer at 30 in.

**Wine Measure***Table of Units*

4 gills	= 1 pint
2 pints (pt.)	= 1 quart
4 quarts (qt.)	= 1 gallon (gal.)

*Use:* commercial liquid measure in the United States. The British Imperial System has slightly different values and equivalents.

*Standard:* the gallon, as specified under Apothecaries' Liquid Measure

<i>Equivalents:</i>	1 quart U. S.	=	57.75 cubic inches
	1 quart U. S.	=	0.946 liter

Other equivalents as per Apothecaries' Liquid Measure.

**Household Measure**

Although containers used for measurements in household cookery are apt to vary considerably, there are certain standards and it is possible to obtain a set of standard spoons as well as a standard cup. Properly used, these instruments are accurate for all practical purposes, including microscopy. A spoon set comprises one each  $\frac{1}{4}$ ,  $\frac{1}{2}$ , and 1 teaspoons, dessertspoon and tablespoon. In using these for measurements of dry ingredients, fill the spoon completely, then level off with the back of a straight-edged knife. Level a cup similarly; if liquid is being measured, fill the cup brimful; only level spoons and cups are considered. Some confusion may arise in the case of tablespoons. The standard is the largest spoon used at the table, generally for serving purposes; the old-fashioned type frequently seen in coin silver, and containing 4 teaspoons. In many modern cookbooks a smaller, more recent type is indicated, holding 3 teaspoons. The proper designations teaspoonfuls, tablespoonfuls, and the like have been shortened in this account to omit the final ending, as is customary today in recipes.

*Table of Units and Equivalents*

1 drop	= 1	minim
1 teaspoon	= 1	fluid dram = 3.7 cubic centimeters
1 dessertspoon	= 2.5	fluid drams
3 teaspoons	= 1	smaller type tablespoon
4 teaspoons	= 1	standard tablespoon = 14.8 cubic centimeters
2 tablespoons	= 1	fluid ounce = 29.6 cubic centimeters
2 fluid ounces	= 1	wineglass = 59.2 cubic centimeters
8 fluid ounces	= 1	cup
16 tablespoons	= 1	cup
2 cups	= 1	pint

**Metric Measure***Table of Units*

1 milliliter (ml.)	= 1	cubic centimeter
1,000 milliliters	= 1	liter = 1 cubic decimeter
100 liters (l.)	= 1	hectoliter (hl.)

*Use:* standard fluid measures in countries employing the metric system, and for scientific work everywhere. The term milliliter is now uncommon, cubic centimeter being used instead.

*Standard:* the liter. This is based on the standard meter and consists of the volume of a cube whose edge is 0.1 meter.

*Equivalents:*

1 milliliter	=	0.061 cubic inch
1 milliliter	=	16.23 minims
1 milliliter	=	0.271 fluid dram
1 milliliter	=	0.034 fluid ounce
1 liter	=	61.028 cubic inches
1 liter	=	2.113 pints U. S.
1 liter	=	1.057 quarts U. S.
1 liter	=	0.908 dry quart U. S.
1 liter	=	2.205 pounds avoirdupois
1 liter	=	2.679 pounds troy

The last two items above convert liters to mass and are figured for distilled water at 4° C.

## MEASUREMENTS OF LENGTH

### English Linear Measure

#### *Table of Units*

12 lines	=	1 inch
12 inches (in., ")	=	1 foot
3 feet (ft.,')	=	1 yard (yd.)

The inch is commonly divided into halves, fourths, eighths, etc., as in commercial operations, or into tenths, hundredths, and thousandths as by machinists. The original division by twelfths (lines) is now seldom used.

*Use:* for linear measurements in English-speaking countries.

*Standard:* the yard. In England, the standard yard is that distance between two lines crossing gold studs set in a platinum bar when measured at 62° F., with the barometer at 30 in. In the United States, the standard yard is defined in terms of the international prototype meter, which is declared to be 39.37 in.

*Equivalents:*

1 inch	=	25.399 millimeters
1 inch	=	2.539 centimeters
1 foot	=	30.48 centimeters
1 foot	=	0.305 meter
1 yard	=	0.914 meter

### Metric Linear Measure

#### *Table of Units*

1,000 microns (mu, $\mu$ )	=	1 millimeter
10 millimeters (mm.)	=	1 centimeter
100 centimeters (cm.)	=	1 meter (m.)

*Use:* standard linear measures in countries employing the metric system, and for scientific work in all countries.



**Standard:** the meter, the prototype preserved at the International Bureau of Weights and Measures, Paris

**Equivalents:**

1 micron	=	0.00003937	inch
1 millimeter	=	0.03937	inch
1 centimeter	=	0.3937	inch
1 centimeter	=	0.033	foot
1 meter	=	39.3708	inches
1 meter	=	3.2809	feet
1 meter	=	1.0936	yards

### MEASUREMENTS OF TEMPERATURE

**Centigrade (C.).**—Zero is the freezing point of water and  $100^{\circ}$  the boiling point, the latter taken at 760 mm. barometric pressure. Used in most European countries and for all scientific work.

**Fahrenheit (F.).**—Zero is the temperature produced by mixing equal quantities of snow and salt; the freezing point of water is  $32^{\circ}$  and the boiling point  $212^{\circ}$ , resulting in a range of  $180^{\circ}$  between these two standard points. Used for all general purposes in the United States and England.

**Reaumur (R.).**—Zero is the freezing point of water and  $80^{\circ}$  the boiling point. Generally outmoded but still used to some extent in France, Germany, and Russia.

**Equivalents:**

1 degree F.	=	$0.55^{\circ}$ C.	=	$0.44^{\circ}$ R.
1 degree C.	=	$1.8^{\circ}$ F.	=	$0.8^{\circ}$ R.
1 degree R.	=	$1.25^{\circ}$ C.	=	$2.25^{\circ}$ F.

#### Conversions:

To convert F. to C., subtract  $32^{\circ}$  and multiply the remainder by  $\frac{5}{9}$ .

$$\text{Formula: } C = \frac{5}{9}(F - 32)$$

To convert C. to F., multiply by  $\frac{9}{5}$  and add  $32^{\circ}$  to the product.

$$\text{Formula: } F = \frac{9}{5}C + 32$$

To convert F. to R., subtract  $32^{\circ}$  and multiply the remainder by  $\frac{4}{9}$ .

$$\text{Formula: } R = \frac{4}{9}(F - 32)$$

To convert R. to F., multiply by  $\frac{9}{4}$  and add  $32^{\circ}$  to the product.

$$\text{Formula: } F = \frac{9}{4}R + 32.$$

To convert C. to R., multiply by  $\frac{4}{5}$ .

To convert R. to C., multiply by  $\frac{5}{4}$ .

## REFRACTIVE INDICES

Refraction, considered in the simplest possible manner, is the bending of a ray of light when it passes obliquely from a medium of one density into another medium of different density, as when a ray enters water from air, or passes from glass into balsam. Refractive Index (R. I.) compares the amount of bending in any given substance with that in a vacuum, which is arbitrarily designated as 1. Since all materials are denser than a vacuum, all R. I.'s are greater than 1.

The figures thus indicate the relative bending powers of the media on light rays and are much used by manufacturers in selecting appropriate glasses for lens making, and by the microscopist when choosing suitable mounting media. The rule is that the nearer the R. I. of the medium to that of the object, the less the contrast and greater the transparency; however, the farther the R. I. of the medium from that of the object, preferably higher than lower, the greater the contrast and less the transparency. Most diatoms, for example, require a medium with an R. I. considerably higher than that of canada balsam in order to bring out by contrast the fine striations and markings on the valves.

Indices are determined by an instrument known as a refractometer, and will vary somewhat according to the source and purity of the sample being tested, as well as for the wave length of light used. For the D line of the spectrum they are approximately as follows:

## ALPHABETICAL LISTING

Acetic acid, glacial.	1.372	Carbolic acid.....	1.549
Acetone.....	1.364	Carbon bisulphide.	1.630
Air.....	1.0003	Cellosolve.....	1.406
Albumen.....	1.350	Chloroform.....	1.446
Alcohol, <i>n</i> butyl...	1.399	Clarite.....	1.544
tertiary butyl...	1.388	Clarite X.....	1.567
ethyl, absolute..	1.367	Colophonium.....	1.545
isopropanol.....	1.378	Creosote.....	1.538
methanol.....	1.329	Damar.....	1.520
methyl.....	1.323	Diamond.....	2.417
normal propyl...	1.386	Diaphane.....	1.483
Anilin.....	1.586	Diatoms, silica....	1.434
Balsam, camsal....	1.478	Dioxan.....	1.423
canada, solid...	1.535	Ether, ethyl.....	1.354
canada, xylene..	1.526	Euparal.....	1.483
damar.....	1.520	Fluorite.....	1.433
Benzene.....	1.503	Glass, crown.....	1.49 -1.55
Calcite, Iceland		barium crown...	1.54 -1.66
spar.....	1.654	fluor crown.....	1.46 -1.51

## ALPHABETICAL LISTING

flint.....	1.54 -1.96	linseed.....	1.485
barium flint.....	1.53 -1.67	olive.....	1.476
borate flint.....	1.53 -1.62	origanum, Cretan	1.494-1.504
Glycerin, 50%.....	1.397	sandalwood.....	1.51
concentrated.....	1.473	thyme.....	1.483-1.510
Gum arabic.....	1.512	turpentine.....	1.474
Gum damar.....	1.520	wintergreen, syn-	
Hyrax.....	1.71 -1.822	thetic.....	1.53
Iceland spar.....	1.654	Paraffin, liquid....	1.471
Isobutyl methacry-		Piperine.....	1.657-1.68
late.....	1.477	Plastic micro cover	
Mersol, F. & G....	1.51	slips, Turttox....	1.528
Methanol.....	1.329	Quartz.....	1.46 -1.55
Methyl benzoate..	1.517	Quinidine.....	1.617
Methyl salicylate..	1.53	Realgar.....	2.14 -2.549
Monobromide of		Sandarac cam-	
naphthalene....	1.658	phloral.....	1.485
Neutral synthetic		Sirax.....	1.81
mountant, F. &		Styrax ( <i>L. orien-</i>	
G.....	1.515	<i>talis</i> ).....	1.582
Oil, almond.....	1.478	Styrax ( <i>L. styraci-</i>	
anilin.....	1.586	<i>flua</i> ).....	1.63
aniseed.....	1.55	Synthetic neutral	
bergamot.....	1.464	mountant, F. &	
cajeput.....	1.46	G.....	1.515
cassia.....	1.578	Synthetic oil of	
castor.....	1.490	wintergreen.....	1.53
cedar, thickened.	1.520	Terpineol.....	1.484
cedar, unthick-		Toluene.....	1.495
ened.....	1.510	Trichlorethylene..	1.478
cinnamon.....	1.619	Water, distilled....	1.336
cloves.....	1.533	sea.....	1.343
lemons.....	1.527	Xylene.....	1.497

## NUMERICAL LISTING

1.0003	air	1.367	ethyl alcohol, absolute
1.323	alcohol, methyl	1.372	acetic acid, glacial
1.329	methanol	1.378	isopropanol
1.336	water, distilled	1.386	normal propyl alcohol
1.343-	water, sea	1.388	tertiary butyl alcohol
1.350	albumen	1.397	glycerin, 50%
1.354	ether, ethyl	1.399	<i>n</i> butyl alcohol
1.364	acetone	1.406	cellosolve

## NUMERICAL LISTING

1.423	dioxan	1.526	canada balsam, xylene
1.433	fluorite	1.527	oil of lemons
1.434	diatoms, silica	1.528	plastic micro cover slips,
1.446	chloroform		Turttox
1.46	glass, fluor crown, low	1.53	glass, barium flint, low
1.46	oil of cajeput	1.53	glass, borate flint, low
1.46	quartz, low	1.53	methyl salicylate (syn-
1.464	oil of bergamot		thetic oil of winter-
1.471	paraffin, liquid		green)
1.473	glycerin, concentrated	1.533	oil of cloves
1.474	oil of turpentine	1.535	canada balsam, solid
1.476	olive oil	1.538	creosote
1.477	isobutyl methacrylate	1.54	glass, barium crown, low
1.478	oil of almonds	1.54	glass, flint, low
1.478	camsal balsam	1.544	clarite
1.478	trichlorethylene	1.545	colophonium
1.483	diaphane	1.549	carbolic acid
1.483	euparal	1.55	aniseed oil
1.483	oil of thyme, low	1.55	glass, crown, high
1.484	terpineol	1.55	quartz, high
1.485	linseed oil	1.567	clarite X
1.485	sandarac camphloral	1.578	oil of cassia
1.490	castor oil	1.582	styrax ( <i>L. orientalis</i> )
1.49	glass, crown, low	1.586	anilin oil
1.494	oil of origanum, Cretan,	1.617	quinidine
	low	1.619	oil of cinnamon
1.495	toluene	1.62	glass, borate flint, high
1.497	xylene	1.630	carbon bisulphide
1.503	benzene	1.63	styrax ( <i>L. styraciflua</i> )
1.504	oil of origanum, Cretan,	1.654	calcite, Iceland spar
	high	1.657	piperine, low
1.510	cedar oil, unthickened	1.658	monobromide of naph-
1.510	oil of thyme, high		thalene
1.51	glass, fluor crown, high	1.66	glass, barium crown, high
1.51	mersol, F. & G.	1.67	glass, barium flint, high
1.51	sandalwood oil	1.68	piperine, high
1.512	gum arabic	1.71	hyrax, low
1.515	synthetic neutral mount-	1.81	sirax
	ant, F. & G.	1.822	hyrax, high
1.517	methyl benzoate	1.96	glass, flint, high
1.520	cedar oil, thickened	2.14	realgar, low
1.520	damar	2.417	diamond
		2.549	realgar, high



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